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**A study of the action of inflammatory
mediators on intracellular calcium in
cultured rat sensory neurones.**

By

Alison Foster

Thesis submitted for the degree of Doctor of
Philosophy to the Faculty of Sciences of the
University of London

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Abstract

Tissue damage releases many endogenous mediators including ATP, serotonin (5-HT), histamine and prostaglandins such as PGE₂. Many of these substances directly excite primary nerve endings to elicit either pain (ATP) or both pain and itch (histamine and 5-HT). Prostaglandins do not directly activate the nerve endings but are known to sensitise them to histamine and 5-HT. Although histamine is known to act on sensory neurones via the H₁ histamine receptor, the receptor subtypes activated by ATP and 5-HT remain unclear. In addition, the mechanism by which prostaglandins sensitise the nerve endings is unknown. These issues have been investigated in cultured rat dorsal root ganglion neurones by using the ratiometric indicator Fura-2 to monitor changes in intracellular calcium.

5-HT evoked a rise in $[Ca^{2+}]_i$ in approximately a fifth of neurones. This proportion remained unchanged when cells were stimulated in a nominally calcium free solution or with the 5-HT₂ receptor agonist, α -methyl 5-HT. Thus, sensory neurones responded to 5-HT via 5-HT₂ receptors. PGE₂ sensitised neurones to 5-HT as shown by a leftward shift in the dose response curves. A similar leftward shift was seen for the effect of PGE₂ on the response to histamine. The effects of PGE₂ could be mimicked by application of forskolin and were blocked by the protein kinase A (PKA) inhibitor, H89. This suggests that the sensitisation results from a phosphorylation reaction mediated by PKA.

Three quarters of DRG neurones responded to ATP and a similar proportion responded to the P₂Y agonist, 2-methyl thio ATP. However, although the response to the P₂Y agonist was reduced by pretreatment with the PLC inhibitor U73122 it was not totally abolished, supporting the notion that P₂X receptors were also involved.

In conclusion, it appears that the calcium responses elicited by histamine and 5-HT in rat sensory neurons are primarily due to activation of G protein coupled receptors and that the sensitivity of these receptors was enhanced by PKA mediated phosphorylation.

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ABBREVIATIONS

5-HT	5-hydroxytryptamine
AC	adenylyl cyclase
AM	acetoxymethyl
AMCA	7-amino-4-methyl-coumarin-3-acetic acid
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Ca ²⁺	calcium ions
[Ca ²⁺] _i	intracellular calcium ions
cAMP	cyclic 3',5'-adenosine monophosphate
[cAMP] _i	intracellular cAMP
CCD	charge-coupled device
CGRP	calcitonin gene-related peptide
CNS	central nervous system
DP	PGD ₂ receptor
DRG	dorsal root ganglion
EGTA	ethylene glycol-bis(b-aminoethyl ether) N,N,N',N'-tetraacetic acid
EP	PGE ₂ receptor
ER	endoplasmic reticulum
FP	PGF _{2α} receptor
FITC	fluorescein isothiocyanate
GDNF	glial cell line derived neurotrophic factor
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IB ₄	isolectin B ₄
IP	PGI ₂ receptor
IP ₃	inositol-1,4,5-trisphosphate
IP ₃ R	inositol-1,4,5-trisphosphate receptor
K ⁺	potassium ions
MCPB	1-(3-chlorophenyl)biguanide
NGF	nerve growth factor
PCR	polymerase chain reaction
PG	prostaglandin
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
TP	TXA ₂ receptor
TRPV1	vanilloid receptor subtype 1
TRITC	tetramethyl rhodamine isothiocyanate
TTX	tetrodotoxin
TXA ₂	thromboxane A ₂
UTP	Uridine 5'-triphosphate

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1.0- Introduction

Nociception refers to the detection of noxious stimuli by the nervous system. Specialised sensory receptors termed nociceptors are activated in the periphery by noxious stimuli. Nociceptors transmit impulses to the central nervous system (CNS), where motor responses are initiated and the sensations of itch or pain are perceived. Itch (Pruritus) is an unpleasant sensation associated with the desire to scratch. Although itch has been written about for centuries it remains poorly understood. Its physiological role, like pain, is to act as a self-protective mechanism to help defend the skin against harmful external agents including parasites and plants.

While most people's experience of the sensation is limited to the occasional insect bite there is a wide diversity of diseases associated with itch. It is a dominant symptom of skin diseases while many systemic diseases, such as chronic renal failure, liver diseases and HIV are also accompanied by persistent or recurring itch attacks (Twycross *et al.*, 2003). Initially the associated scratching and pinching has an antipruritic effect (Greaves and Wall, 1998). However, these actions can cause tissue damage, which in turn can lead to infection and further medical problems. The scratching can also evoke the release of inflammatory mediators that potentially induce or aggravate itch resulting in an itch-scratch cycle.

The development of effective treatments for itch have been hampered by the lack of reliable models resulting in the lack of detailed knowledge about target cells and the neural pathways involved. Due to the highly subjective nature of itch, the sensation is difficult to study objectively in man and there are currently few reliable animal models. Humans can distinguish the distinct features of the itch and pain sensations, thus are able to quantify the intensity of itch itself (Magerl *et al.*, 1990). Two broad approaches are used to directly measure itch: subjective recording using a visual scale or experimental psychophysical technique. In the first technique pruritic stimuli are applied and subjects rate the level of perceived itch using a visual scale. However, this technique has fixed end points, a limited range of measurements and does not give any information on neural pathways or signalling molecules. Perceptual matching techniques are used for psychophysical assessment of itch in subjects. An electrical stimulus is applied to the skin of the finger and the subject halts the stimulus when the amplitude of the sensation matches the intensity of the itch experienced during histamine iontophoresis in the inside of the forearm. This approach is not limited by visualised predetermined limits and gives a continuous indication of intensity. Much of the early work on itch was thus collected from human subjects using psychophysical studies.

In animal models, itch has to be measured by a behaviour that is only elicited by pruritic stimuli and not by other sensory stimuli. Scratching in animals is a regular occurrence (e.g. grooming) and thus may not be a true indicator of itch. However, a recent behavioural study showed that intradermal injections into the

rostral part of the back of the mouse showed that pruritic but not algogenic stimuli elicited scratching at the injected site by the hind paw (Kuraishi *et al.*, 1995). This approach may be of value in examining different treatments for itch.

1.1.0- Early studies of itch, pain and inflammation

1.1.1- The triple response

When tissue is damaged a vascular response occurs which consists of three independent parts (Lewis, 1927). This response was termed the triple response, which consists of:

1. A local area of redness appears immediately at the site of injury due to local dilatation of the arterioles.
2. A red flare spreading over several centimetres rapidly develops around the injury.
3. As the flare subsides, an area of oedema develops at the site of the injury due to the leakage of fluid from the post capillary venules.

The area of inflammation becomes sensitive to innocuous stimuli such as clothing rubbing which is termed hyperalgesia. Lewis *et al* (1927b) first observed that this hyperalgesia not only occurred within the injured tissue (primary hyperalgesia) but extended into the region of the flare and beyond into the normal tissue outside the injured area (secondary hyperalgesia). Primary hyperalgesia is thought to result from a decrease in the firing threshold of peripheral terminals of primary afferent neurones and thus mediated peripherally (LaMotte *et al.*, 1982, LaMotte *et al.*, 1983,). Within this area of secondary

hyperalgesia innocuous stimuli such as light stroking produce pain (allodynia) while noxious stimuli produce abnormally intense and prolonged pain. Pruritic stimuli can also induce a secondary allodynia where light tactile stimulation evokes itch (Graham *et al.*, 1951). LaMotte *et al* (1991) showed that secondary hyperalgesia could be prevented by anaesthetic blockade of peripheral nerves innervating the site of capsaicin application. This evidence suggests that secondary hyperalgesia is mediated by central sensitisation. A more detailed overview of the mechanisms involved in the induction of neurogenic inflammation and the accompanying hyperalgesia is given below (p.21).

1.1.2- Identification and study of itch and pain spots

In 1922, Von Frey demonstrated that the sensory modalities including itch and pain had a punctate distribution in the skin. Using a hair-like plant bristle mounted on a handle he was able to map out the skin for sensory spots uniquely sensitive to itch. Using electrical stimulation the pruritic nature of the itch spots was confirmed (Bishop, 1943). However, both the electrical and mechanical experiments demonstrated that weak stimuli at these spots gave rise to itch while stronger stimuli at the same spots produced pain. Shelley and Arthur (1957) correlated the physiological activation of these spots with the histology of the skin. Using a modification of the classical methylene blue nerve stain they studied large superficial shavings of skin in which active or inactive cowhage (*Mucuna pruriens*) spicules remained as a marker. Subsequently the skin biopsies showed that itch and pain spots possessed a greater number of fine

free nerve endings (Von Frey, 1922, Shelley and Arthur, 1957) than the neighbouring areas. The early experiments also used various methods of chemical stimulation to induce pain and itch (e.g. iontophoretic application of irritants to the skin, intradermal injection, pricking the skin through a drop of agonist). The conjunctival sac was even used to record the irritant effects of histamine and demonstrate the mode of action of capsaicin. Nevertheless, many of these early techniques did not produce reliable and reproducible results.

In 1964 Keele and Armstrong introduced the cantharidin blister technique. Cantharidin, the active component of cantharides, is an anhydride of cantharidic acid which occurs in blister beetles. Cantharidin was applied to the skin for four to six hours. After removal, a red area was left which over time developed into a blister. The fluid and raised epidermis was removed and algogenic or pruritic agents were applied to the blister base. Acetylcholine, 5-HT and ATP evoked pain when applied to an exposed blister base. The latency between application and onset of pain varied with mediator. Acetylcholine provoked an immediate pain upon application while 5-HT and plasma kinins evoked a sensation of pain only after a period of time. Exposure of a blister base to histamine and agents that liberate histamine (e.g. compound 48/80) evoked itch with a delayed onset. Thus, the blister base experiments demonstrated that a number of chemicals could be applied to the exposed base to evoke reproducible sensations of itch or pain. However, this technique raised the issue of sensitisation, a key feature of itch and pain. Shelley and Arthur (1957) believed that the cantharidin blister

technique was unsuitable for the study of pruritic agents because the nerve endings were already sensitised prior to any agonist application. The sensitised nerve endings in an exposed blister base would in fact resemble those at the site of tissue damage or inflammation.

To overcome this limitation, Shelley and Arthur (1957, 1955, Arthur and Shelley, 1955) used the spicules of cowhage to study the itch spots. Their technique allowed minute quantities of fluid to be injected at different depths into the epidermis. They demonstrated that the spicules of cowhage themselves contained a pruritic which proved to be a proteolytic enzyme termed mucunain. The insertion of boiled spicules into the skin did not elicit itch, so they could be impregnated with different test solutions and used to investigate peripheral aspects of itch. A range of compounds including histamine, trypsin and papain were shown to activate itch spots, clearly establishing the importance of chemical mediators in the process of itch (Arthur and Shelley, 1955, Shelley and Arthur, 1957). Subsequently, it has been found that pruritus can be evoked in the skin directly by mechanical and thermal stimuli or indirectly through chemical mediators (see Table 1.1, p.14). These mediators can elicit itch by stimulating the nerve endings directly e.g. histamine and papain (protease) or by causing mast cell degranulation thus releasing histamine e.g. compound 48/80.

Table 1.1 – Stimuli that elicit or augment itch

Physical	Mechanical	Touch, Pressure
	Thermal	Warming
Chemical	Non Specific Irritants	Acids, Alkalis
	Inflammatory Mediators	Histamine, 5-HT, Prostaglandins
	Histamine Releasing Substances	Compound 48/80
	Peptidases	Trypsin
	Neuropeptides	Substance P
	Opioids	Morphine

(adapted from McMahon and Koltzenburg, 1992).

1.1.3- Is itch a separate sensation?

The close relationship between itch and pain has led many to contend that itch is a subliminal form of pain (e.g. Von Frey, 1922, Lewis *et al.*, 1927a). While itch and pain share some features they are distinct sensory modalities and can be distinguished in the following ways. While pain can be elicited in most tissues, itch sensations can only be elicited in the mucous membranes, the superficial layers of the skin and the conjunctiva. Arthur and Shelley (1959) showed that the removal or destruction of the epidermis abolished itch but not cutaneous pain. Moreover, behavioural studies have shown differences in the protective reflexes elicited by itch and pain: itch evokes the scratch reflex, while cutaneous pain leads to the withdrawal reflex. Differences also occur in their treatment as the pain relieving non-steroidal anti inflammatory drugs (e.g. indomethacin and aspirin) cannot alleviate itch and systemic administration of opioids actually potentiates itch (Ballantyne *et al.*, 1988). Nevertheless, itch induced by the injection of a cowhage spicule on the forearm can be abolished by a pinprick applied locally. Therefore, painful stimuli can mask itch and the two sensations

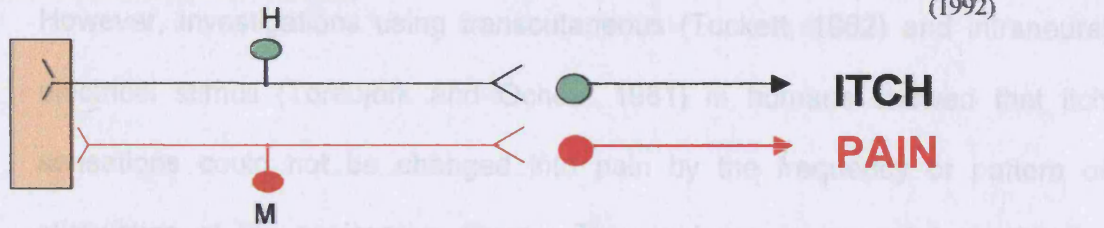
rarely coexist in the same skin region (Bickford, 1938, Graham *et al.*, 1951, McMahon and Koltzenburg, 1992, Greaves and Wall, 1996, Ward *et al.*, 1996). To conclude, the evidence supports the notion that itch is a unique sensory experience that is qualitatively distinct from pain.

Early work failed to identify a subset of primary afferent fibres or specific mechanisms that could explain all the features of itch and pain. In consequence, four rather different theories of itch have been proposed (see Figure 1.1):

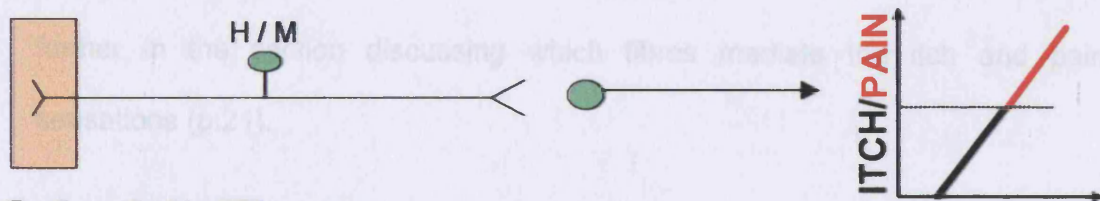
1. The specificity theory proposes that there are itch specific neurones i.e. a group of afferent fibres and central neurones exists that only respond to pruritic stimuli.
2. The intensity theory hypothesizes that both itch and pain are mediated by the same set of neurones and the sensation perceived is due to the intensity of the stimuli. Low activity in the nociceptive fibres signals itch while higher activity signals pain.
3. The selectivity theory suggests that pain and itch sensations are mediated by specific subsets of fibres. Thus itch sensations are encoded by a specific set of afferents having different central connectivities to those signalling pain.
4. The pattern theory suggests primary afferents are not specific for either sensation but instead itch is encoded by the temporal or spatial firing of the afferents.

Specificity Theory

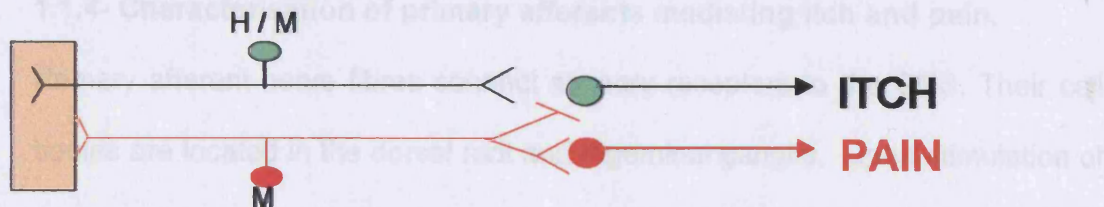
Adapted from McMahon and Koltzenburg
(1992)



Intensity Theory



Selectivity Theory



Skin Primary Afferents Central Neurons

Figure 1.1 – Three of the proposed neural pathways for itch.

The afferent neurones responsive to histamine are labelled H, while M indicates the afferent neurones sensitive to noxious agents such as mustard oil (adapted from McMahon and Koltzenburg 1992).

However, investigations using transcutaneous (Tuckett, 1982) and intraneural electrical stimuli (Torebjork and Ochoa, 1981) in humans showed that itch sensations could not be changed into pain by the frequency or pattern of stimulation of the nociceptive fibres. This evidence is inconsistent with the intensity and pattern theories. The remaining two theories will be discussed further in the section discussing which fibres mediate the itch and pain sensations (p.21).

1.1.4- Characterisation of primary afferents mediating itch and pain.

Primary afferent nerve fibres connect sensory receptors to the CNS. Their cell bodies are located in the dorsal root and trigeminal ganglia. Upon stimulation of the sensory receptors, information is transmitted to the CNS by trains of nerve impulses in primary afferent nerves. The properties of these fibres differ markedly depending on the sensory modality. The primary afferent fibres can be classified into three main types on the basis of their diameter, structure and conduction velocity (see Table 1.2). Nociceptors are a subpopulation of peripheral afferent fibres that are activated by noxious stimuli. Nociceptors are unusual sensory cells in that they respond to a wide spectrum of physical (heat, and cold), chemical (acid, irritants and inflammatory mediators) and mechanical (stretch and pressure) stimuli. The heterogeneous nature of nociceptors is reflected in the neurotransmitters they utilise, the receptors and ion channels they express, their conduction velocities and their ability to be sensitised during inflammation, injury and disease. However, nociceptors are only activated at stimulus intensities that are capable of causing tissue damage.

Table - 1.2 – Classification of the primary afferent neurones.

Fibres	Diameter	Conduction Velocities (ms ⁻¹)	DRG neurones	DH laminae innervated	Sensory function
A α + β	Large (>10 μ M) Myelinated	Fast 30-100	Large Type A	III-VI	Proprioception vibration and touch
A δ	Medium (2-6 μ M) myelinated	Intermediate 12-30	Medium	I/II _o III-V	Thermoreception (cold), touch and pain
C	Small (0.4-1.2 μ M) Unmyelinated	Slow 0.5-2.0	Small Type B	I/II _o IV/V, X	Thermoreception (warm), touch, pain and itch

Capsaicin is an irritant compound in the capsicum plant that is capable of producing an intense burning pain when intradermally injected into humans (LaMotte *et al.*, 1992). Electrophysiological studies showed that low concentrations of capsaicin excited specific subsets of sensory neurones including almost all C fibre polymodal and thermo nociceptors and some A δ fibre polymodal nociceptors (Heyman and Rang, 1985). These fibres are also excited by bradykinin and low pH. Thus capsaicin sensitivity has proven to be an extremely useful functional marker for nociceptive fibres (Fitzgerald, 1983, Holzer, 1991).

The thinly myelinated A δ fibres elicit a rapid first phase of pain, sharp in nature while unmyelinated C fibres evoke a second wave of dull pain. The following evidence suggests that itch, like pain, is primarily mediated by unmyelinated C fibres. The differential blocking of the myelinated fibres in the skin does not abolish histamine-induced itch (Handwerker *et al.*, 1987). The induction of itch after iontophoretic application of histamine to the skin is characteristically slow

(30-60 seconds after the stimulus) (Schmelz, 2001). The destruction of the unmyelinated nerve endings by pre-treatment with capsaicin (Simone *et al.*, 1996) abolished the histamine-induced itch (Handwerker *et al.*, 1987). When C fibre conduction was blocked by peripheral nerve compression, itch was abolished (Handwerker *et al.*, 1987). This evidence clearly suggests itch is mediated by unmyelinated C fibres.

Microneurography is used extensively to study discharge patterns of human cutaneous C fibres. This technique showed that nociceptive C fibres are heterogeneous. They can be classified by their responsiveness to chemical, mechanical and thermal stimuli (Bessou and Perl, 1969, Torebjork and Hallin, 1974). C fibres that are excited by Von Frey hair stimulation are termed mechano-responsive (CM). Thermo-responsive C fibres (CH) are excited by noxious radiant heat stimuli. However, some C fibres are responsive to more than one stimulus. The most common type of C fibres are excited by noxious chemical, mechanical and thermal stimuli and thus are referred to as polymodal fibres (Bessou and Perl, 1969, Beck *et al.*, 1974, Lynn and Carpenter, 1982, Torebjork, 1974). They are strongly activated by mustard oil (Tuckett and Wei, 1987, Handwerker *et al.*, 1991) but are either insensitive to or very weakly activated by, histamine (Handwerker *et al.*, 1991). The response pattern of polymodal C nociceptors does not match the prolonged itch sensations induced by intradermal injection of histamine (Simone *et al.*, 1987) or by iontophoretic application of histamine (Handwerker *et al.*, 1987, Magerl *et al.*, 1990). This

evidence suggests that polymodal C fibres mediate pain yet itch is unlikely to be mediated by this same subset of C fibres.

Using a refined marking microneurography technique a new subset of mechano-insensitive C fibres (CM_i) has been identified in human skin (Schmidt *et al.*, 1995). A subgroup of these mechanoinsensitive C fibres was found to be sensitive to histamine (Schmelz *et al.*, 1997). The responses of these C fibres to iontophoretic application of histamine into the skin (Schmelz *et al.*, 1997) parallel the itch rating of the subjects (Magerl *et al.*, 1990). It has been proposed that these fibres mediate the itch response in humans.

Recently in cat, the iontophoretic application of histamine to the skin was shown to selectively excite a class of lamina 1 spinothalamic tract neurones. The responses of these neurones parallel the pure itching sensation induced by histamine in humans (Magerl *et al.*, 1990) and match the responses of the peripheral histamine-sensitive mechano-insensitive nociceptive C fibres (Schmelz *et al.*, 1997, Andrew and Craig, 2001).

The evidence reviewed above suggests that different subsets of C fibres mediate the two sensations. The discovery of histamine-sensitive mechano-insensitive C fibres provides evidence for a pathway specific for the processing of itch (Schmelz *et al.*, 1997, Andrew and Craig, 2001,). Nevertheless, although the peripheral mechano-insensitive nociceptors showed sustained responses to a number of pruritic agents, they were additionally excited by some algogenic

substances e.g. bradykinin, capsaicin (Schmelz *et al.*, 2003). Therefore this new subset of C fibres suggests that some afferents are excited by both pruritic and algogenic stimuli. This evidence argues against the specificity theory which states that there are itch specific neurones. However, it supports the selectivity theory, which proposes that the histamine sensitive afferents also respond to algogenic agents. Differentiation between the sensations reflects the central processing.

1.1.5- Neurogenic Inflammation

In 1901, Bayliss first reported that the electrical stimulation of the dorsal roots evoked vasodilatation which was unaffected by sympathectomy or division of the dorsal root between the ganglia and spinal cord. He postulated that these neurones not only conduct afferent information to spinal cord but also served an efferent function (Bayliss, 1901). As mentioned earlier, injury to the skin results in the triple response characterised by redness at the site of injury followed by a flare spreading over several square centimetres. This is followed by the development of local oedema at the site of injury.

Lewis (1927) believed that the spread of the flare was due to a local axon reflex, which involves the antidromic activation of connecting nerve fibres that innervate the adjacent skin. This notion is supported by the following evidence: activation of the axon collaterals release neuropeptides from their terminals. These act on target cells (e.g. mast cells, arteriolar smooth muscle) to produce a range of effects including mast cell degranulation, vasodilatation, (visible as flare

surrounding the site of injury) and plasma protein extravasation (which gives rise to a wheal). These are collectively termed neurogenic inflammation (Jancso *et al.*, 1967, Holzer, 1988, Geppetti and Holzer, 1996). Although nociceptive fibres contain a variety of neuropeptides, most of the evidence suggests that calcitonin gene-related peptide (CGRP) and substance P appear to be the main initiators of neurogenic inflammation (Holzer, 1988). Direct application of substance P to peripheral tissue produces vasodilatation, increased vascular permeability (Lembeck and Holzer, 1979) and degranulation of mast cells. The degranulation of the mast cells results in the local release of histamine (Ebertz *et al.*, 1987). This is consistent with the observation that the injection of histamine into the skin mimics almost exactly the triple response (Lewis, 1927). CGRP also evokes vasodilatation and potentiates the plasma extravasation induced by other inflammatory mediators e.g. substance P and histamine (Brain *et al.*, 1985, Gamse and Saria, 1985). The administration of substance P or CGRP agonists mimics the symptoms of neurogenic inflammation while the receptor antagonists and antibodies attenuated them (Lembeck *et al.*, 1982, Couture and Cuello, 1984). Recent studies have suggested that other mediators released during tissue damage (e.g. nerve growth factor) may also play a role in the development of inflammation (Lewin *et al.*, 1994; Koltzenburg *et al.*, 1999).

1.1.6- Sensitisation induced by inflammatory mediators

Hyperalgesia occurs when non-noxious stimuli are perceived as painful or when the intensity of pain perceived from noxious stimuli increases. Nociceptor

sensitisation is thought to underlie the phenomenon of peripheral hyperalgesia. It is characterised by lower firing thresholds for nociceptive activity, increased spontaneous activity and increased frequency of firing to suprathreshold stimuli (Meyer *et al.*, 1994). Sensitisation is triggered by a number of extracellular mediators released from damaged or inflamed tissue.

During neurogenic inflammation a large number of biologically active substances are released. A number of these substances are known to sensitise the peripheral nerve endings to stimulation by other mediators. To induce peripheral sensitisation these mediators can either act directly on primary afferent nerves or indirectly by stimulating other non-neuronal or neuronal cells. These cells then release hyperalgesic agents that can then act on primary afferent nerves. These mediators include bradykinin, ATP, 5-HT and many products of the arachidonic acid pathway (e.g. prostaglandin E₂ (PGE₂)). Although bradykinin, ATP and 5-HT are primarily known for their potent algogenic properties they also have a secondary action that can induce peripheral sensitisation.

Inflamed tissue often contains high concentrations of prostaglandins. The activation of phospholipase A₂ generates arachidonic acid, which in turn is metabolised by cyclo-oxygenases to produce various prostaglandins. The application of PGE₂ to an exposed blister base failed to evoke pain and intradermal injections of prostaglandins alone do not normally elicit pain (Ferreira, 1972). However, early animal studies showed that prostaglandins

reduced the intensity of noxious stimuli needed to elicit various escape behaviour (Ferreira *et al* 1978). In agreement, Hamilton *et al* (1999) showed that animals pre-treated with prostaglandins required lower concentrations of ATP for nocifensive responses. In man, peripheral sensitisation is observed as a heightened perception of pain (Handwerker and Reeh, 1991, Handwerker and Kobal, 1993).

These behavioural observations are supported by both *in vivo* and *in vitro* experiments, which show that the co-administration of a nociceptive agent with prostacyclin (PGI₂) or PGE₂ causes a dramatic increase in the responsiveness of the neurones (Ferreira *et al.*, 1978, Baccaglini and Hogan, 1983). Prostaglandins are known to act directly on peripheral terminals of nociceptive afferents to produce hyperalgesia (Taiwo and Levine, 1989). The elimination of all known indirect pathways such as inhibition of cyclo-oxygenase pathways of arachidonic acid metabolism, depletion of sympathetic postganglionic neurones and depletion of polymorphonuclear leukocytes did not affect the hyperalgesia to mechanical stimuli induced by PGE₂. The prostaglandins are thought to produce their effects by binding to specific receptors on the sensory neurones enabling them to lower the firing threshold of these neurones (Schaible and Schmidt 1988). The mechanisms involved in the prostaglandin-induced sensitisation will be explored in the discussion.

1.2.0 - The role of histamine, 5-HT and ATP in the sensations of itch and pain.

After tissue damage or inflammation a large number of mediators are released into the extracellular milieu. The early work of Shelley and Arthur (1957, 1959) and Keele and Armstrong (1964) demonstrated that histamine, 5-HT and ATP could evoke the sensations of itch and pain. This study is primarily concerned with the actions of these mediators on the intracellular concentration of calcium in primary sensory neurones. Below is a description of the tissue sources, the actions in the periphery and the receptors for each of these important inflammatory mediators.

1.2.1- Histamine

Histamine is found in nearly every mammalian tissue with particular abundance in the skin, lungs, gut and gastric mucosa. Histamine is formed by decarboxylation of the amino acid histidine by the enzyme L-histidine decarboxylase (Tanaka and Ichikawa, 2001). In the peripheral tissues, histamine is stored in mast cells, basophils, enterochromaffin cells and some neurones. In mast cells and basophils it is stored in intracellular secretory granules loosely bound to heparin and chondroitin-4-sulphate (Soll *et al.*, 1981, Beaven *et al.*, 1983). The concentration of histamine in these secretory granules is in the molar range (Beaven *et al.*, 1983). Inflammatory and chemical stimuli can trigger the release of histamine from mast cells by producing a rise in intracellular calcium, which in turn activates a calcium dependent exocytosis (Lagunoff *et al.*, 1983) resulting in the degranulation of the mast cell. Following

degranulation, the histamine concentration in the extracellular space adjacent to the mast cell is in the region of 15-30 mM although it falls progressively with time (Nicolson *et al.*, 2002). However, those nerve endings in close proximity (<100 nm) to degranulating mast cells (Dimitriadou *et al.*, 1997) will experience millimolar concentrations of histamine.

The injection of histamine into the skin almost exactly mimics the triple response (Lewis, 1927). Lewis (1927) also noted that the triple response induced by histamine was frequently accompanied by itch. Many other investigators have since confirmed the pruritogenic action of histamine by a number of different methods. The application of histamine to an exposed blister base (Keele and Armstrong, 1964) and the intraepidermal injection of an inactivated cowhage spicule coated with histamine (Shelley and Arthur, 1957) both induced itch. Nowadays, histamine is frequently used in experimental models of itch as iontophoretic application onto the skin elicits a pure and almost graded sensation of itch (Magerl *et al.*, 1990). Histamine is firmly established as a potent pruritic as well as a major mediator of inflammation.

Histamine Receptors

The membrane histamine receptors have been classified into four distinct subtypes (H₁, H₂, H₃ and H₄) all of which are G protein coupled (see Table 1.3). All the receptors have been cloned: H₁ and H₂ in 1991 (Gantz *et al.*, 1991, Yamashita *et al.*, 1991), H₃ in 1999 (Lovenberg *et al.*, 1999) and H₄ in 2001 (Nguyen *et al.*, 2001, Zhu *et al.*, 2001). The histamine receptor subtype

mediating the calcium response in DRG neurones has previously been characterised (Nicolson *et al.*, 2002). Here, a brief summary of the histamine receptor subtypes is given (see Table 1.3).

Table 1.3 – Classification of the histamine receptor subtypes.

Receptor Subtype	G protein	Transduction Mechanism	Location
H ₁ R	G _q	Ca mobilisation	Widely distributed PNS+CNS
H ₂ R	G _s	Activates adenylyl cyclase	Stomach ,Heart ,cells of immune system
H ₃ R	G _{i/o}	Inhibits adenylyl cyclase	Limited to brain
H ₄ R	G _{i/o}	Inhibits adenylyl cyclase	Bone marrow, leukocytes, small intestine

Application of histamine to rat DRG neurones elicits a rise in calcium in a small sub-population of sensory neurones. This calcium response is blocked by the application of H₁ receptor antagonist, mepyramine, but not by the application of antagonists of H₂ receptor (cimetidine) or H₃ receptor (thioperamide) (Nicolson *et al.*, 2002). Thus, the H₁ receptor elicits a rise in intracellular calcium in cultured sensory neurones.

1.2.2- Serotonin (5-HT)

The monoamine 5-HT, like histamine, is widely distributed throughout the body. It is synthesised from tryptophan, which is derived from dietary sources. The prominent sources of 5-HT in the periphery are platelets and mast cells in rats

and platelets in humans (Graziano, 1988). Circulating levels of free 5-HT in plasma are low since platelets accumulate 5-HT by active transport and store it in high concentration. 5-HT is one of the mediators in the inflammatory soup released from platelets, mast cells, and basophils that infiltrate an area of tissue damage. The following evidence supports the role of 5-HT in inflammation, hyperalgesia, itch and pain.

In the skin, iontophoretic application of 5-HT induces wheal, flare and itch. Itch and the vasocutaneous reactions induced by 5-HT were significantly weaker than those induced by iontophoretic application of histamine (Weisshaar *et al.*, 1997). Moreover, intradermal injections of 5-HT provoke a weaker sensation of itch than induced by histamine (Fjellner and Hagermark 1979).

Early studies showed that application of 5-HT to an exposed blister base gave rise to a sharp but transient pain sensation (Keele and Armstrong, 1964, Richardson *et al.*, 1985). Moreover, intradermal injections of 5-HT evoked pain (Lindahl, 1961, Keele and Armstrong, 1964). 5-HT has long been recognised as a potent excitant of afferent fibres in a wide variety of peripheral nerves (Wallis *et al.*, 1982, Christian *et al.*, 1989). Electrophysiological studies show that 5-HT induces pain due to the excitation of unmyelinated C fibres (Beck and Handwerker, 1974). It has long been believed that the direct activation of nociceptive fibres is via the activation of 5-HT₃ receptors as pain induced by application of 5-HT to an exposed blister base can be abolished by application of 5-HT₃ antagonists (Richardson *et al.*, 1985). However, more recent studies

have suggested that 5-HT₂ receptors may also be involved. Discharge of C fibres elicited by 5-HT are inhibited by the 5-HT₂ receptor antagonist, ketanserin, but not by the 5-HT₃ receptor antagonist, MDL72222 (Grubb *et al.*, 1988). This evidence suggests the involvement of multiple receptor types in the mediation of pain. Application of 5-HT ligands was shown both to hyperpolarize (Todorovic and Anderson, 1992) and to depolarise (Molokanova and Tamarova, 1995) sensory neurones. The effects produced were dependent on the population of neurones stimulated and on the selectivity of the ligand applied. The variation in the responses seen in DRG neurones could also be explained by the interaction of 5-HT with multiple receptor subtypes.

5-HT seems to have a number of different roles in peripheral pain transduction. Bleehen and Keele (1977) showed that it acts synergistically with adenosine to induce pain when applied onto an exposed blister base. Furthermore, when a low dose of 5-HT was combined with other inflammatory mediators and injected into the hind paw of an animal it induced a synergistic increase in paw lifting and licking-features characteristic of a pain response (Hong and Abbott, 1994). This effect was observed even when the co-administrated mediator was not able to induce a pain response by itself (Hong and Abbott, 1994). This evidence strongly supports a role for 5-HT in the potentiation of pain produced by other inflammatory mediators.

A further action of 5-HT is the induction of hyperalgesia to thermal and mechanical stimuli. Injections of 5-HT into the foot pad of rats reduced the paw

withdrawal latency to radiant heat (Tokunaga *et al.*, 1998). 5-HT can also sensitise the primary afferent terminals to mechanical stimuli. This hyperalgesic effect was dose dependent (Taiwo and Levine, 1992). The onset of hyperalgesia was rapid, suggesting that 5-HT was acting directly on the primary afferent neurones. This was confirmed when the elimination of all known indirect pathways such as blockage of cyclo-oxygenase pathways of arachidonic acid metabolism, depletion of sympathetic postganglionic neurones and depletion of polymorphonuclear leukocytes failed to attenuate the mechanical hyperalgesia induced by 5-HT. This is consistent with the notion that hyperalgesia can be evoked by a direct action of 5-HT on the peripheral nerve endings.

The evidence reviewed above illustrates the range of different effects produced by 5-HT in the periphery. Seven families of 5-HT receptors have been identified (Hoyer *et al.*, 1994). All except one (5-HT₃) of the 5-HT receptors couple to G protein cascades that modulate the intracellular concentration of second messengers. To acquire a better understanding of the range of effects mediated by 5-HT, there is a need to investigate the multiple 5-HT receptors expressed on primary afferent fibres. It is important to understand how these receptors interact with each other and other inflammatory mediators. The characteristics of the 5-HT receptor subtypes which appear to be involved in nociception will be detailed, in conjunction with my results, in the discussion.

5-HT plays a complex role in many of the features of nociception in the central nervous system. In the spinal cord 5-HT is released primarily from the raphe

spinal serogeneric pathway, which contributes to the descending inhibition of spinal pain transmission. The released 5-HT inhibits the responses of neurones in the dorsal horn to noxious stimuli, producing an analgesic effect. Thus the central actions of 5-HT produce analgesia, while it has an excitatory action on peripheral nociceptors.

1.2.3- Adenosine triphosphate (ATP)

ATP is an essential metabolite involved in energy transfer in all cells. It is present in millimolar concentrations in the cytosol of all cell types. Cytoplasmic ATP is utilised for driving many pumps (e.g. Na/K ATPase, Ca-ATPase), for GTP synthesis and by many G proteins and various protein kinases. Apart from the cytoplasmic and mitochondrion stores, ATP is present in synaptic vesicles of some nerve terminals where it is co-localised with acetylcholine and noradrenaline. Extracellular levels of ATP are normally maintained in the nM range, which are achieved by the expression of ecto-nucleotidase in most cells. These enzymes can sequentially degrade ATP to ADP, AMP, adenosine and inosine within cells. Nevertheless, upon tissue damage ATP is released from a variety of sources including primary nerve afferents, sympathetic nerves, endothelial and epithelial cells.

Over thirty years ago, Burnstock first proposed the notion of purinergic transmission, which initially met with much resistance. Non adrenergic non cholinergic nerves are now known to utilise ATP as a transmitter when supplying the smooth muscle of the intestine and bladder (Burnstock *et al.*, 1972, 1978).

Once ATP was established as a neurotransmitter there was considerable interest into its possible role in pain. ATP is one of the inflammatory mediators released in the milieu during inflammation or tissue damage. Bleehen and Keele (1977) showed that exogenously applied ATP to an exposed blister base induced pain. The actions of ATP as a potent algogen were confirmed when intracutaneous injections (Hilliges *et al.*, 2002), intradermal injections (Burnstock and Wood, 1996, Coutts *et al.*, 1981) and iontophoretic application to the skin (Hamilton *et al.*, 2000) all evoked an intense pain. In behavioural studies, injections of ATP into the hind paw of rats resulted in dose-dependent nocifensive behaviour (Bland-Ward and Humphrey, 1997, Hamilton *et al.*, 1999). In humans, ATP was found to activate a sub population of C nociceptors and some A δ fibres (Hilliges *et al.*, 2002, Schmelz *et al.*, 2003). Moreover, ATP has been shown to elicit fast excitatory responses in primary afferent fibres (Krishtal *et al.*, 1983, Lewis *et al.*, 1995). This evidence supports the notion that ATP is a mediator of pain at primary afferent nerve endings.

Extracellular ATP activates receptors collectively called P₂ purinoceptors (Burnstock and Kennedy, 1985). They are distinct from the P₁ receptors that are activated by the ubiquitous endogenous purine adenosine. The P₂ purinoceptors can be subdivided into two major classes: P₂X (which are ligand gated cation channels) and P₂Y (which are G-protein coupled receptors) (Abbracchio and Burnstock, 1994). A large number of ATP receptors have been identified and there is considerable debate as to the receptors involved in

mediating pain and hyperalgesia. The P₂ receptors will be further reviewed in the discussion.

1.2.4- Prostanoids

Arachidonic acid is a ubiquitous component of cells. It is generated from membrane phospholipids via activation of phospholipase A₂ (Shimizu and Wolfe, 1990). Arachidonic acid is metabolised by two different enzymes, cyclo-oxygenases give rise to various prostanoids and lipoxygenases give rise to various leukotrienes (Rang *et al.*, 1995). In response to tissue injury, prostaglandins are synthesised *de novo* from arachidonic acid. The prostaglandins produced in the early stage of inflammation play a role in the dilatation of blood vessels. This leads to the development of the redness, warmth and oedema characteristic of inflamed tissue. Prostaglandins also play a vital role in the hyperalgesia that accompanies inflammation.

There are five naturally occurring prostaglandins PGD₂, PGE₂, PGF_{2α}, PGI₂ and TXA₂ which activate the receptors DP, EP, FP, IP and TP respectively (Coleman *et al.*, 1994) (see Table 1.4). PGE₂ and PGI₂ are the predominant prostaglandins generated in response to injury or inflammation. All prostanoids activate several second messenger pathways.

Prostaglandins are known to sensitise primary afferent fibres to some inflammatory mediators. Early animal studies showed that the sensitising

effects of PGE₂ could be mimicked with cAMP analogues (Ferreira and Nakamura, 1979). Previous work in animal studies showed that the co-injection of 5-HT and PGE₂ enhanced the sensation of pain (Hong and Abbott, 1994). Recent studies have begun to elucidate the mechanisms underlying this sensitisation.

Table 1.4 - Classification of the prostaglandin receptor subtypes.

Receptor	Activating Prostaglandin	Transduction System
D ₂	PGD ₂	↑ cAMP via G _s (↑ AC)
EP ₁	PGE ₂	↑ [Ca ²⁺]
EP ₂		↑ cAMP via G _s (↑ AC)
EP ₃		↓ cAMP via G _i inhibit AC
EP ₄		↑ cAMP via G _s (↑ AC)
FP	PGF _{2α}	↑ PI + Ca via G _q
IP	PGI ₂	↑ cAMP via G _s (↑ AC)
TP	TXA ₂	↑ PI turnover via G _q

AC – adenylyl cyclase; PI inositol tris phosphate;

In cultured DRG neurones pre-treatment with PGE₂ potentiated the bradykinin evoked increase in calcium in small diameter cells and increased the number of cells that responded to low concentrations of bradykinin (Smith *et al.*, 2000). The sensitising effects of PGE₂ on the calcium response could also be mimicked by the membrane permeant cAMP analogue, dibutyl cAMP, and inhibited by H89, inhibitor of cAMP dependent protein kinase A (PKA) (Smith *et al.*, 2000). Previous work by Nicolson (2000) demonstrated that when rat DRG neurones were pre-treated with PGE₂ there was a significant increase in cAMP levels.

Subsequently it was shown that pre-treatment of neurones with PGE₂ could sensitise the response to histamine. Agents that inhibited cAMP production could block this sensitisation (Nicolson, 2000). Thus suggesting cAMP had a role in this sensitisation. However, it was not clear whether cAMP was acting directly or indirectly through the activation of PKA.

1.2.5- Activation of G protein linked receptors by inflammatory mediators

Noxious stimuli are detected by sensory receptors at peripheral nerve terminals of nociceptive fibres. Excitation causes a depolarisation of the nerve endings that elicits action potentials in the afferent fibres. Here DRG neurones have been used as a model for afferent fibre terminals (see section 1.3.0). They express various types of channels that can be directly activated including stretch activated channels and voltage gated channels. Additionally there are ligand-gated channels, where activation either leads to the activation of an ion channel directly linked to the receptor (e.g. 5-HT₃, P₂X, TRPV1) or activation of a G protein linked cascade.

Capsaicin binds to specific vanilloid receptor subtype 1, TRPV1, expressed on the peripheral terminals of nociceptive fibres (Caterina *et al.*, 1997). Additionally protons and noxious heat can activate the TRPV1 channel. Upon activation, a non-selective cation channel opens allowing the influx of calcium and sodium ions resulting in depolarisation of the cell. Action potentials are fired transmitting impulses to the CNS where pain and a burning sensation are perceived.

Activation of G proteins can modulate channel function by direct binding to parts of the channel protein, or activate various second messenger transduction pathways (McKnight, 1991). A single activated receptor can activate multiple G protein molecules thus amplifying the ligand binding event. The G proteins are classified by their α subunits proteins of which four classes exist based on amino acid sequence comparison (Neer, 1995, Simon *et al.*, 1991) (see Table 1.5).

Table 1.5 – Classes of G protein α subunits.

Class	Members	Effectors	Signalling Molecules
α_s	α_s, α_{olf}	\uparrow adenylyl cyclase calcium channels	\uparrow cAMP \uparrow Calcium Influx
α_i	$\alpha_{i-1}, \alpha_{i-2}, \alpha_{i-3}$ $\alpha_{0A}, \alpha_{0B}, \alpha_{11},$ α_{12}, α_z	\downarrow adenylyl cyclase, K^+ channels, activate cGMP phosphodiesterase	\downarrow cAMP
$\alpha_q/11$	$\alpha_q, \alpha_{11}, \alpha_{14},$ α_{15}, α_{16}	Phospholipase C β	\uparrow IP ₃
α_{12}	α_{12}, α_{13}	Regulate Na^+/K^+ exchange Cl ⁻ channels	

The \uparrow arrows indicate an increase while the \downarrow arrow indicates a decrease.

PGE₂ activates the G protein coupled EP receptors that can activate the adenylyl cyclase / cAMP pathway. The EP receptors activate different G α subunits. EP₂ and EP₄ receptors activate the stimulatory G α subunit thus increase adenylyl cyclase activity and hence increase cAMP levels. While activation of EP₃ receptor coupled to the inhibitory G α subunit leads to a

decrease in adenylyl cyclase activity and thus decreased cAMP levels. Cyclic AMP can activate protein kinases, which catalyses the phosphorylation of serine and threonine residues in a variety of cellular proteins and hence regulates their function (Cohen, 1992).

Another pathway commonly utilised in DRG neurones is the inositol-1,4,5-trisphosphate(IP₃)/ phospholipase C (PLC) cascade (Berridge and Irvine, 1989). Activated H₁ receptors interact with a G protein coupled to the PLC /IP₃ transduction pathway to stimulate the mobilisation of calcium from IP₃ sensitive internal stores and the activation of protein kinase C (PKC).

Almost all calcium signalling systems function by generating brief increases in intracellular calcium concentration [Ca²⁺]_i, which activate a variety of cellular activities such as channels (e.g. calcium gated potassium channel) and enzymes (e.g. calcium/calmodulin-dependent protein kinases) (Berridge, 1997, Berridge *et al.*, 2003). The cell has two sources of calcium to initiate signalling: an infinite extracellular source and a finite intracellular source. The endoplasmic reticulum (ER) is a low capacity, high affinity calcium store while the mitochondria represent a high capacity, low affinity calcium store. Three main types of channels regulate calcium entry via the plasma membrane: voltage operated channels, store operated channels and receptor operated channels. Each class of channel has different kinetic properties with the voltage gated channels and receptor operated channels providing brief high intensity bursts of calcium while the store operated channels have small but sustained influx of

calcium (Berridge, 1997). At least five major types of voltage activated calcium channels are expressed on sensory neurones (L, N, P/Q, T and R). These channels vary in their distribution and activation properties (Scroggs and Fox, 1992, Scott *et al.*, 1991). The store operated channels main function is to replenish and maintain internal calcium stores (Berridge, 1995, Putney *et al.*, 2001). When the internal stores are full little calcium enters through these channels. Immediately the stores content of calcium begins to decline calcium entry by capacitative calcium entry through these channels is promoted. Thus store-operated channels are controlled by the state of filling of the internal stores.

Internal stores play a crucial role in the generation and maintenance of calcium signals (Simpson *et al.*, 1995, Berridge, 2002). Two channels mediate the release of calcium from the ER stores: the IP₃ receptor gated channel and the ryanodine-gated channel (Bezprozvanny *et al.*, 1991, Ehrlich *et al.*, 1994). A critical feature of both receptor channels is their sensitivity to cytosolic calcium. The receptors gating the release of calcium from internal stores is controlled by calcium itself. This enables calcium released from one receptor to excite its neighbour thereby regulating a regenerative calcium wave further amplifying the signal. Both channels display positive and negative regulation by calcium (Berridge, 1997). The generation and dynamics of calcium signals in response to receptor activation by algogenic and pruritic mediators can be monitored using calcium indicator dyes such as Fura 2.

1.3.0- Cultured sensory neurones as a model for the study of peripheral aspects of nociception.

Dorsal root ganglia contain the cell bodies of the primary afferent neurones that relay nociceptive and proprioceptive information from the periphery to the spinal cord. Due to the difficulty of studying peripheral nerve endings *in situ*, primary cultured DRG neurones have been commonly employed to investigate many aspects of sensory transduction. This approach assumes that the receptors they express are similar to those operating on the primary nerve endings. The following evidence shows that many of the characteristics of nociceptors are expressed by sensory neurones in culture:

- Immunostaining with an anti-neurofilament marker, RT97, showed that isolated small diameter dark DRG neurones were neurofilament poor (Lawson, 1979, Lawson *et al.*, 1984, Perry *et al.*, 1991). *In vivo* they give rise to the slow conducting unmyelinated C fibres and thinly myelinated A δ fibres (Harper and Lawson, 1985a) that are known to transmit nociceptive information.
- Specific algogenic agents activate DRG neurones maintained *in vitro*. Capsaicin, a known excitant of nociceptors, also evokes responses in a substantial proportion of these small diameter neurones. Moreover, low concentrations of bradykinin also activate some cells (Baccaglini and Hogan 1983).
- Prostaglandins can sensitise peripheral nociceptors to other noxious stimuli. In the presence of PGE₂ a number of cultured sensory

neurones showed a significant increase in the number of action potentials elicited by high potassium (Baccaglini and Hogan, 1983).

- Immunostaining has shown that many cultured DRG neurones contain substance P (Baccaglini and Hogan, 1983). In vivo substance P is released by capsaicin sensitive fibres and plays an important part in the induction of inflammation and pain (Holzer, 1988).

From this evidence we may conclude that cultured DRG neurones are useful for the study of aspects of sensory transduction mechanisms that are inaccessible to analysis *in vivo*, particularly the actions of algogens and pruritics.

The various types of DRG neurones differ in their intrinsic electrophysiological (Harper and Lawson, 1985b, Scroggs and Fox, 1992), cytochemical (Dodd *et al.*, 1984, Schoenen *et al.*, 1989) and anatomical properties (Lawson, 1979). Two thirds of the small DRG neurone population are characterised as nociceptive primary sensory cells (Perry and Lawson, 1991).

During development all small sensory neurones require nerve growth factor (NGF) for survival (Snider and McMahon, 1998). However, the sensitivity of these cells to neurotrophic factors changes as the animal matures. In cultures from adult animals the small diameter cells can be divided into two major subpopulations by their sensitivities to neurotrophic factors (Averill *et al.*, 1995, Michael *et al.*, 1997, Molliver *et al.*, 1997, Bennett *et al.*, 1998). About 40-45% of the population express the NGF receptor tyrosine kinase A. These neurones

coexpress CGRP and substance P. As noted earlier (p.22) both these neuropeptides are released from sensory fibres and are known initiators of inflammation and pain. As a further third of small diameter DRG neurones mature, they downregulate the expression of tyrosine kinase A receptors and increase the expression of another trophic factor receptor tyrosine kinase *Ret*, which is activated by glial cell derived neurotrophic factor (GDNF). Thus, these two populations can be differentiated by their sensitivity to GDNF. They also have distinct histochemistry, expressing the enzyme thiamine monophosphatase (TMP) and possess cell surface glycoconjugates that can be identified by binding of isolectin IB₄ (Silverman and Kruger, 1990, Averill *et al.*, 1995, Molliver *et al.*, 1997). Therefore, anti CGRP antibody and IB₄ binding are generally regarded as markers for NGF and GDNF responsive primary sensory neurones.

1.4.0- Aims of the study

In view of the evidence presented above concerning the possible role of histamine, 5-HT and ATP in itch and pain it is clearly important to characterise the effects of these agents on primary sensory neurones as a prelude to establishing their actions on the sensory nerve endings. Moreover, as some prostaglandins sensitise the skin to agents that evoke itch and pain, it is desirable to investigate the actions of these prostaglandins on the chemical sensitivity of the primary sensory neurones. To achieve these objectives this study has been focused on the following questions:

1. What population of sensory neurones express H_1 receptors? Is receptor expression confined to cells that give rise to unmyelinated nerve fibres?
2. It is known that the response of sensory neurones to histamine can be sensitised by certain prostaglandins (specifically PGE_2 and PGD_2). It is also known that these prostaglandins act to raise intracellular cAMP levels in sensory neurones and that other interventions that raise cAMP also sensitise the sensory neurones to histamine. It is not, however, clear whether this effect of cAMP is mediated via protein kinase A or via some other pathway. Can the sensitisation be blocked by inhibitors of protein kinase A?

3. What fraction of the sensory neurones population is sensitive to 5-HT, determined by its ability to raise intracellular Ca^{2+} ? Which receptors are involved in this response? Can the response be sensitised by prostaglandins in the same way as the responses to histamine? If so, what mechanisms are involved?

4. What fraction of the sensory neurones population is sensitive to ATP, judged by its ability to raise intracellular Ca^{2+} ? Which receptor subtypes are involved (P_2X or P_2Y)? A further, but unfulfilled, goal was to determine whether the response to ATP could be sensitised by prostaglandins.

2.0 Material and Methods

2.1 Primary Culture of Adult Wistar rat DRG neurones

Coverslip Preparation

Coverslips were placed in a glass beaker, covered with 100% ethanol and gently agitated for 10 minutes making sure all coverslips were exposed to ethanol. The ethanol was poured off and the coverslips washed three times in a large volume of double distilled water then autoclaved in distilled water. Under sterile conditions the water was poured off and the coverslips were agitated gently in a solution of poly-L-ornithine (500 µg/ml). After an hour, the poly-L-ornithine was removed and the coverslips were washed three times in a large volume of sterile distilled water. The coverslips were divided and placed in small tubes (approximately 40 per tube) containing distilled water and stored in the fridge until required. Before cells were plated, the coverslips were placed in a 6 well plate (2 per well) and covered with a laminin solution (5µg/ml) for a few hours.

Dissection

Adult Wistar rats (approximately 200g) were killed by exposure to increasing concentrations of CO₂. An incision was first made at the caudal end of the spinal column which was then isolated and removed. The spinal column was then cut laterally down both sides (see Figure 2.1). The dorsal root ganglia located between the vertebrae were carefully dissected out from all levels of the spinal cord and placed in a 35 mm petri dish containing culture medium.

Approximately forty ganglia were removed from the spinal cord. Where appropriate, the roots and processes were carefully removed from the ganglia and the cleaned ganglia were placed in a 35 mm petri dish containing culture medium and collagenase (type IV approx 463U/mg) then left to incubate for 3 hours (37°C, 3% CO₂).

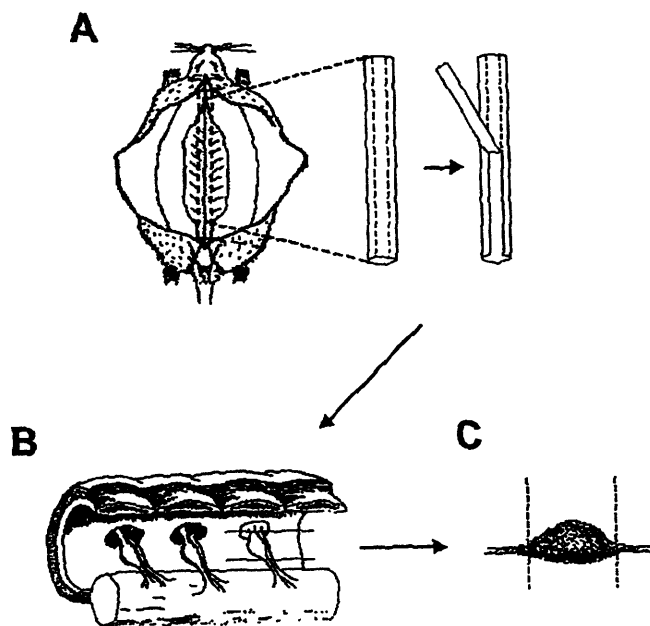


Figure 2.1 – Schematic representation of the DRG dissection procedure.

A) The removal of the spinal column allowing lateral cuts on each side to reveal the ganglia. B) Careful cutting of the ventral and dorsal processes free the ganglia. C) The cleaning (removal of ventral and dorsal processes) of ganglia.

Adapted from (Lindsay *et al.*, 1991).

Dissociation

After incubation, the ganglia were transferred to a centrifuge tube and washed three times in culture medium to remove the collagenase. The ganglia were then resuspended in culture medium and the ganglia were mechanically broken apart by gentle trituration through a flame polished short bore pasteur pipette approximately five times until a cloudy cell suspension was obtained. The remaining ganglia were allowed to settle, and then the supernatant was removed and retained in a centrifuge tube. This process was repeated until the cell to debris ratio in the suspension was low. The removal of the supernatant after each trituration prevents mechanical damage to those cells that had already been dissociated. The collected supernatant was then centrifuged with 13% bovine serum albumin at 1200 rpm for 12 minutes. After centrifugation the supernatant was removed and the cell pellets were gently resuspended in culture medium. The neurones were counted, with a typical yield given between 180,000-240,000 neurones per adult rat (~40 ganglia) then plated appropriately. Prior to plating, the solution in the 6 well plates was aspirated to remove the laminin solution. A drop of cell suspension was placed centrally on the coverslip. For calcium imaging experiments, cells were plated on the glass coverslips coated with poly-L-ornithine and laminin at a density of approximately 0.1×10^6 cells/coverslip. For immunohistochemistry cells were placed at a density of approximately 0.05×10^6 cells/coverslip.

The plated cells were left to incubate for one hour before the coverslips were flooded with culture medium containing NGF (200ng/ml). NGF is need for the

growth of neurones (Johnson *et al.*, 1980, Levi-Montalcini, 1987). Adult DRG can survive in culture without NGF. However, their sensitivity to some chemical mediators has been shown to depend on its presence e.g. capsaicin (Bevan and Winter, 1995). The cells were kept incubated (5% CO₂, 37°C) for one to three days prior to experimentation. However, the majority of experiments were carried out on days one and two.

2.2 - Intracellular calcium imaging

Intracellular calcium changes in response to agonist stimulation were recorded from cultured DRG neurones loaded with the ratiometric dye Fura-2. The Fura-2 dye was excited at 340 and 380 nm and the emitted fluorescence was collected via a cool charged coupled device (CCD) camera system (see page 49)

Dye Loading

Fura-2 is made cell permeant by the substitution of acetoxymethyl ester (AM) groups onto the dye. Although this form is not fluorescent, cells readily take up the acetoxymethyl ester. Once inside the cell an endogenous esterase removes the acetoxymethyl groups and the free dye is fully fluorescent again. The Fura-2AM was dissolved in 50 µg/µl of dimethyl sulfoxide (DMSO). To facilitate the loading of the cells, the detergent pluronic acid (0.01%), was then added to the DMSO/Fura solution and sonicated. The Fura-2AM ester and pluronic acid solution was added to the cell culture medium and sonicated. The cell culture medium was removed from the coverslip and replaced with the cell culture

medium containing Fura-2AM (10 μ M) solution. The cells were returned to the incubator (37°C, 5% CO₂) for 40 minutes. After this time, the coverslip was washed with culture medium and incubated for a further 10 minutes.

The Setup

The plated coverslip was placed in a pre-greased chamber and a Teflon ring placed on top to form a tight seal (see Figure 2.2). An epi-fluorescence microscope (inverted Nikon Diaphot) with a fluor x20 objective lens was used. This objective lens allows adequate transmission of the excitation wavelength to the sample and collection of the fluorescent emission from the sample (see Figure 2.2). To obtain a bright field image of the sensory neurones, a phase contrast lens was used. The fluorescence excitation was provided by a 75W xenon short arc lamp, which provides an even spectrum of illumination through the near ultra violet (UV) and visible ranges. To prevent photolytic damage to the sample, neutral density filters were placed in the path of the UV light. Fura-2 is a dual excitation dye so optical filters are needed to select the necessary 340 nM and 380 nM excitation wavelengths. The filters were located in a rotating wheel controlled by the computer to allow synchronisation of filter changes with image frame acquisition. The filter wheel (Sutter Instruments) was located in the excitation path between the arc bulb housing and microscope (see Figure 2.2). A shutter was placed in the light path to prevent continuous radiation of the sample. A dichroic beam splitter was used to reflect the short wavelengths (340 nM and 380 nM) that excite the preparation whilst allowing through the longer

wavelengths (above 510 nm for Fura 2) emitted as fluorescence by the sample (see Figure 2.2). A 420nm barrier filter was used to block any scattered light from the excitation filters. The emitted fluorescence was collected by a cooled CCD camera (Digital Pixel, Brighton). A computer controlled the camera and filter wheel allowing rapid data acquisition and storage of data.

Cooled CCD Camera

The emitted fluorescence is collected by a cooled charged coupled device (CCD) camera. The image area (sensor) on the CCD camera is made up of rows of wells. When the CCD sensor is exposed to light the photons penetrate the silicon creating hole electron pairs. This charge is stored in each of the potential wells. As more photons fall on the device more electrons are liberated and stored. After a certain collection period the charge stored in the wells is read out into an electronic register. Each well is equivalent to a pixel. The signal produced is proportional to the amount of charge from each pixel. The electron charge is then amplified, sampled and digitised before being displayed and stored. The final image was produced by the balance of intensities of the pixels.

Experiment Protocol

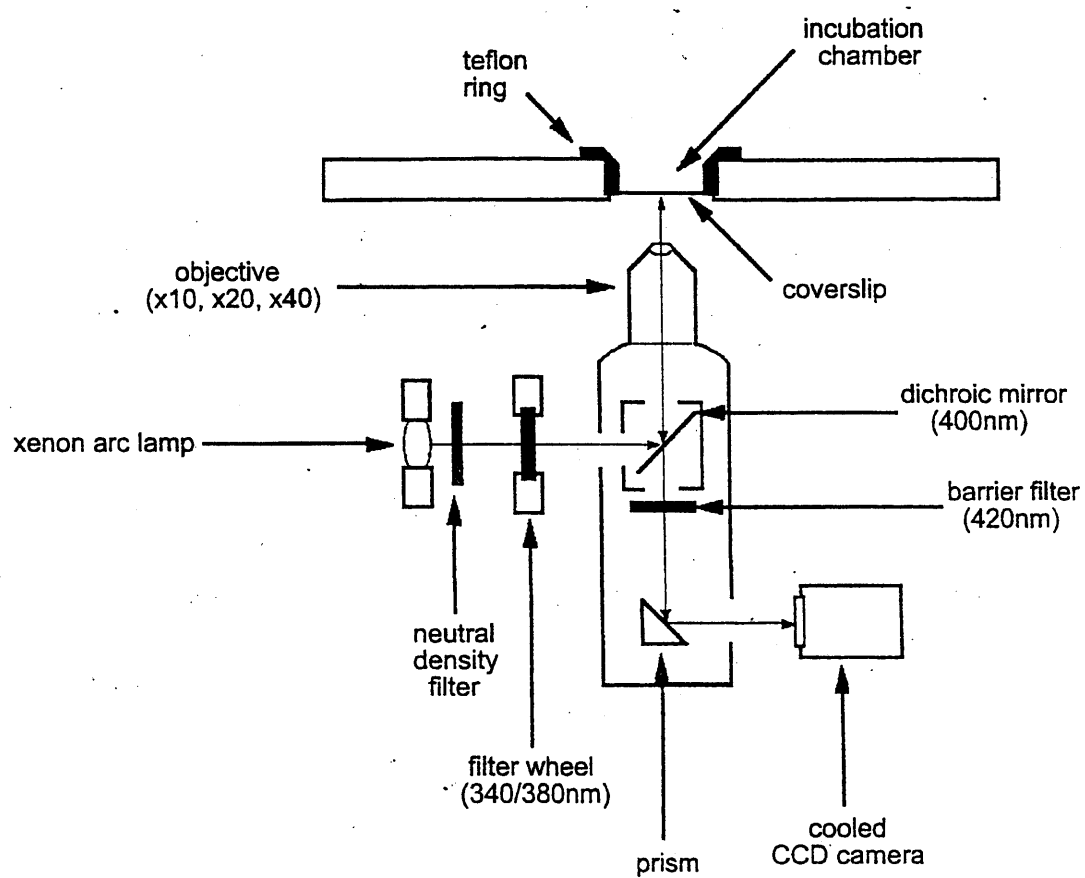
After loading the cells with Fura-2, the coverslip was placed in a pre-greased chamber and a Teflon ring placed on top to form a tight seal (see Figure 2.2). The well was then filled with Na⁺ locke solution (200/400 µl). The chamber was

placed on the microscope stage and a suitable field of cells was selected. A typical field would contain ten to fifteen neurones. A brightfield image of this field was taken and saved to allow cell identification at the end of the experiment. Subsequently the experiment was carried out in the dark to minimise the signal contamination by ambient light. First, a two frame grab was acquired. A grab consists of one frame at 340 nM excitation and one frame at 380 nM excitation with a interval of five seconds between images. The collection time for the 340 nM excitation was three times longer than the time for 380 nM excitation. This initial image acquisition was carried out to check that the signal at each wavelength was neither saturated nor too dim. Ideally the signal was at least three times the value of the background. Neutral density filters with limited transmittance and alteration of the collection time were used to optimise the signal to background ratio. The image pairs were acquired every five seconds and stored on a computer. Subsequently, the ratio of the 340/380 images could be calculated to give an indication of the intracellular free calcium concentration during the course of the experiment. At the end of each experiment the cells were lysed with 0.1% triton to discharge the cytosolic dye. A short sequence of images were taken for the background signal which was due partly to light scatter and partly to dye bound to intracellular constituents.

After the cells were loaded, a suitable field was found and optimal signals obtained. A typical run of 40-70 frames was then acquired (see Figure 2.3). The agonist solutions were added in equal volume to the bath fluid to give the required agonist concentration. This gave instantaneous mixing. The agonist

Figure 2.2 – Schematic representation of the experimental apparatus for fluorescence measurements.

The Fura-2 loaded cells plated on a 16 mm coverslip were mounted on the stage of an inverted epi-fluorescence microscope. The filter wheel rotates between the 340 nm and 380 nm filters to excite the Fura-2. Fluorescent emissions above 420 nm passed through the dichroic mirror and were collected by the CCD camera.



solutions were pipetted into the bath at the appropriate frame (e.g. frame 45). Ten frames were acquired prior to agonist stimulation to allow the calculation of the basal level of calcium. The cells were washed by draining the chamber and replacing the fluid in the bath with a fresh solution. In some protocols the neurones were pre-treated with antagonists or sensitising agents prior to the agonist challenge. The pre-treatment either occurred for a short period (e.g. 45s) within the same sequence of images or, for longer periods in a separate sequence. This was done to assess whether the added agent evoked a response in the cell or affected basal calcium levels. At the end of all experiments, the cells were challenged with 50 mM potassium to check cell viability and identity. Healthy cells respond with a large rise in intracellular calcium upon stimulation with high potassium (see Figure 2.4). Each image sequence was saved at the end of the experiment. All experiments were carried out at room temperature (20-24°C).

Data Analysis

The images were saved to magneto-optical disk as four dimensional image sequences. They were subsequently analysed offline using Kinetic imaging LUCIDA (version 3.5) and Microcal Origin (version 6). First, the image sequences were divided into their respective 340 nm and 380 nm wavelengths and the background for each wavelength was subtracted. The brightest image in the stack of the 340 nm wavelengths was thresholded to give a mask. This mask was then used to set the pixels in the background to zero. The masked

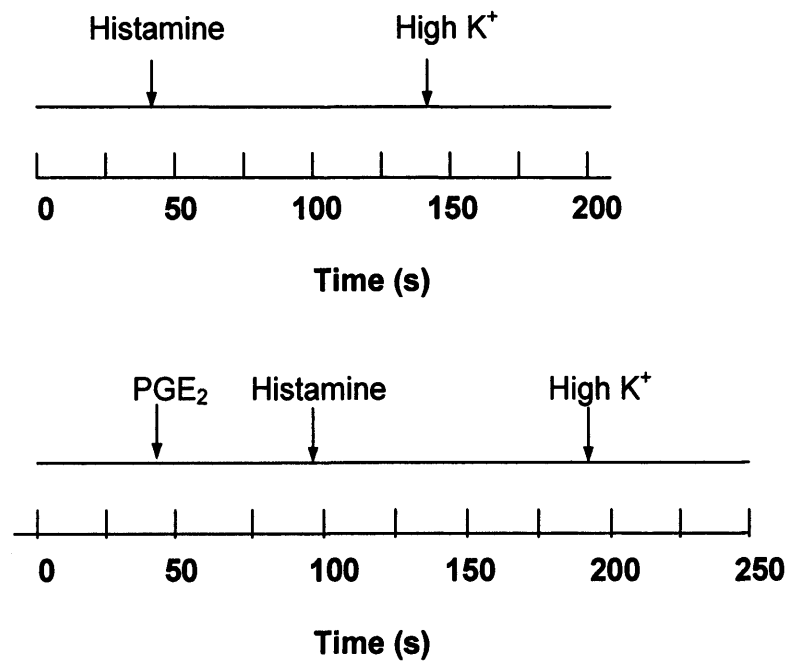


Figure 2.3 – Experimental protocols for the addition of agonists to sensory neurones.

The top line diagram shows the addition and duration of exposure to the agonist. The bottom line diagram shows a protocol for the addition of a sensitising agent before the agonist, in this case histamine. The addition of high potassium (50 mM) in both experiments was to test the viability and identity of the cells.

340 nM image sequence was then divided by the subtracted 380 nM image to give the final ratio image. The cells of interest were marked as regions of interest and the ratio values plotted as a function of time. To be considered healthy, the cells should have a stable basal 340/380 ratio level between 0.8-1.50 and the cells should respond to a challenge of high potassium with a large increase in $[Ca^{2+}]_i$. In the early experiments the basal 340/380 ratio level was slightly lower around 0.6-1.3 due to a difference in the optics of the system. For each individual cell, the mean basal level \pm standard deviation was determined for the frames before the addition of any agonist. The classification of a positive response to any agonist was determined as a rise in intracellular calcium, which was greater than two standard deviations above the mean basal level for that cell. Figure 2.4 shows a typical range of calcium responses elicited by DRG neurones, which have been pre-treated with PGE_2 then challenged with 10 μM 5-HT. The responses of cells 1 and 3 show a rise in intracellular calcium, greater than 2 standard deviations above their baseline, upon stimulation with 5-HT and high potassium. Although there is a slight rise in the intracellular calcium response of cell 2 upon the addition of 5-HT, the increase is not greater than 2 standard deviations above its baseline. Thus, only cells 1 and 3 would be counted as a positive response in this study. The time course of cell 5 shows no response to the challenge with 5-HT however a large rise in intracellular calcium is evoked upon the addition of high potassium. Thus, cell 5 is considered viable and classified in this study as a non-responder to 5-HT (i.e. insensitive to 5-HT). Any cell, which does not respond to the agonist or high potassium challenge,

was considered to be unhealthy or non neuronal and excluded from the study (e.g. Cell 4 in Figure 2.4).

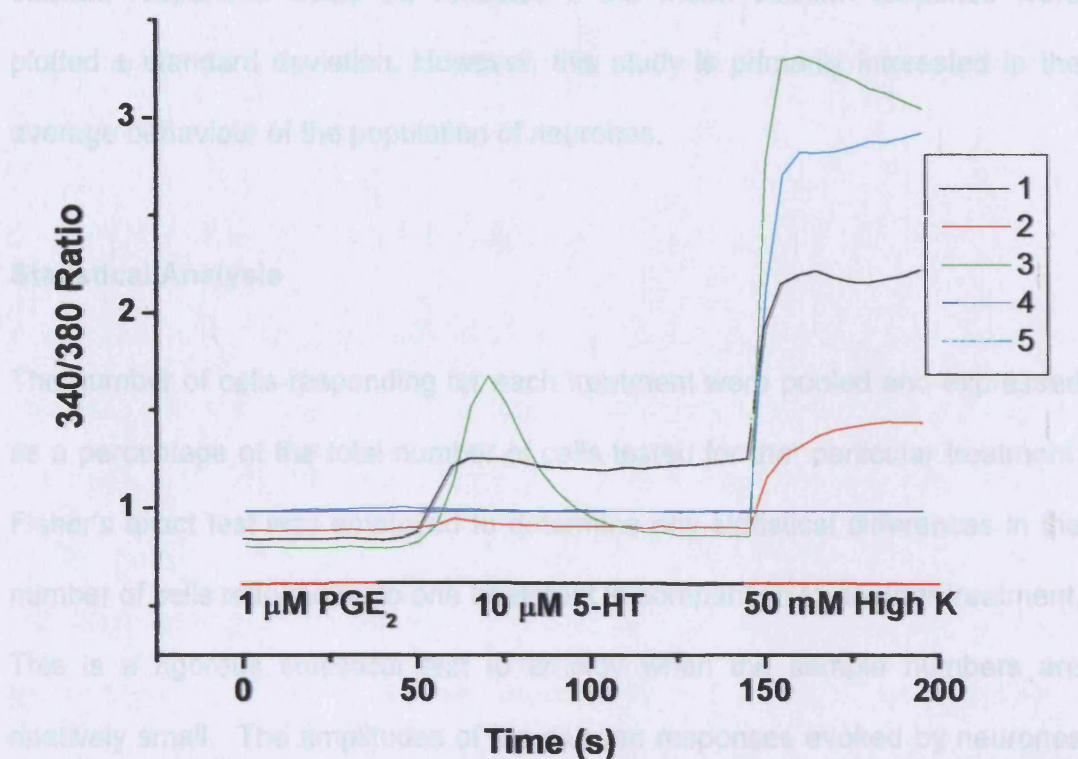


Figure 2.4 – Typical range of calcium responses evoked by neurones, which have been pre-treated with PGE₂ then stimulated with 5-HT and high potassium.

To study the effects of various agonists on the behaviour of the population of neurones, individual responses acquired by the same protocols were pooled and a mean ratio value \pm the standard error of the mean (s.e.m.) was determined. Only the responding cells were included in the mean plots. The individual patterns of cells responses to the agonists were also examined before plotting

the mean calcium responses. Note that the variation in the individual patterns can be masked when plotting the mean \pm s.e.m. The variance in the individual calcium responses would be reflected if the mean calcium response were plotted \pm standard deviation. However, this study is primarily interested in the average behaviour of the population of neurones.

Statistical Analysis

The number of cells responding for each treatment were pooled and expressed as a percentage of the total number of cells tested for that particular treatment. Fisher's exact test was employed to determine any statistical differences in the number of cells responding to one treatment in comparison to another treatment. This is a rigorous statistical test to employ when the sample numbers are relatively small. The amplitudes of the calcium responses evoked by neurones upon stimulation by particular agonists were compared using an unpaired Welch corrected t test.

Calibration of intracellular calcium

Experiments were carried out to allow the calibration of the 340/380 ratio to give an estimation of the free calcium concentration in the cells. Initially the cells were permeabilised with an ionophore, ionomycin (2 μ M), in a medium containing excess calcium (3 mM). This provides values for the maximum ratio at the 340 nm wavelength and a minimum value at the 380 nm wavelength. The cells were washed with a solution containing 0 mM calcium, 10 mM EGTA and

ionomycin which gives the minimum ratio value. After ten minutes the cells were again challenged with the high calcium solution (see Figure 2.5).

The changes in intracellular calcium were calibrated using the following equation (Grynkiewicz *et al.*, 1985).

$$[Ca^{2+}]_i = K_d [(R - R_{min}) / (R_{max} - R)] (S_{f2} / S_{b2})$$

where R is the measured ratio of interest. The R_{min} and R_{max} are the ratios recorded with the zero and high calcium solution respectively. S_{f2} and S_{b2} are the signals at 380 nm wavelength in the zero and high calcium solutions respectively. K_d is the dissociation constant of the indicator and reflects the indicator's affinity for calcium. The K_d value used for Fura 2 was 224 nM (Grynkiewicz *et al.*, 1985). The values obtained in the sensory neurones were R_{min} 1.03 ± 0.08 and R_{max} 5.85 ± 0.59 . The S_{f2}/S_{b2} ratio was calculated as 3.26 ± 0.25 . The average basal ratio in the sample (n=6) of DRG neurones was 1.26 ± 0.08 which according to the above equation would equate approximately to a basal intracellular calcium concentration of 40 nM. However, these calculated values are considerably lower in comparison to the values found previously in rat sensory neurones using the same setup (Nicolson, 2000). The possible reasons for these low values are discussed in section 4.1. Table 2.1 gives an indication of the relationship between the 340/380 ratio and the intracellular calcium concentration.

Table 2.1 – The variation in 340/380 ratio correlates to the concentration of free intracellular calcium.

340/380 Ratio	Free Calcium Concentration ~nM
1.2	25
1.4	60
1.6	100
1.8	140
2.0	180
2.5	320
3.0	500
3.5	760

The values used to calculate the free calcium concentration were: K_d – 224 nM, Sf_2/Sb_2 – 3.26, R_{min} – 1.03 and R_{max} – 5.85.

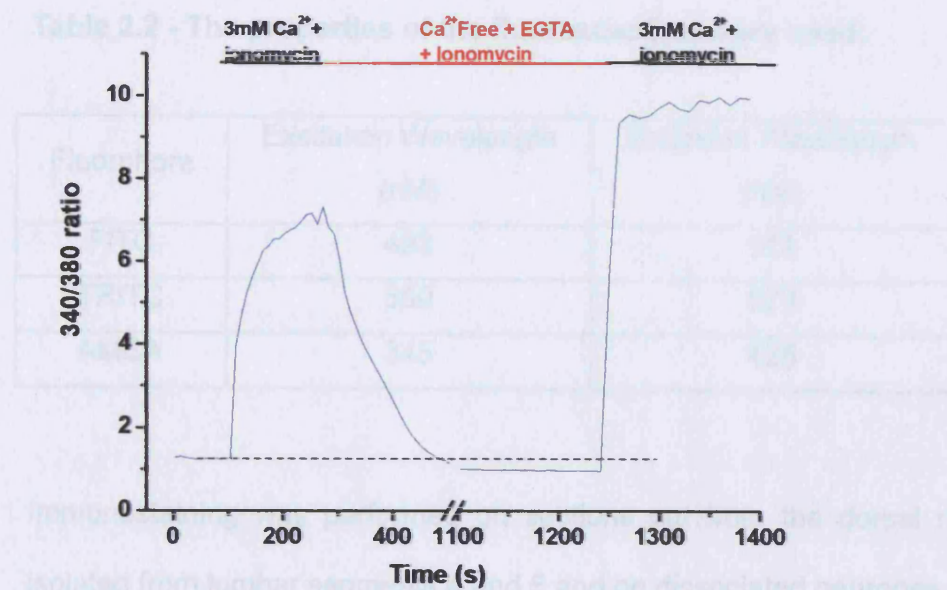


Figure 2.5 – A typical pattern for the ratio changes seen when a cell permeabilised with ionomycin and subsequently challenged with solutions containing a high calcium concentration followed by a solution nominally calcium free.

2.3 - Immunohistochemistry

Antibodies can be used as histochemical reagents when linked to suitable markers. Of the many dyes tested as markers, only fluorescent dyes provide the required sensitivity. The markers must not interfere with the antigen-antibody interaction, be stable upon storage and the peak of the emission spectra should permit its separation from the exciting wavelength. The excitation and emission wavelengths for the markers used in fluorescence immunostaining are given in Table 2.2.

Table 2.2 - The properties of the fluorescent markers used.

Fluorophore	Excitation Wavelength (nm)	Emission Wavelength (nm)
FITC	493	519
TRITC	550	573
AMCA	345	425

Immunostaining was performed on sections cut from the dorsal root ganglia isolated from lumbar segments 4 and 5 and on dissociated neurones.

Fixation and tissue preparation

Prior to immunohistochemistry the dissociated cultured cells and sections were fixed. The culture medium was removed from the plated coverslips, which were then covered in 4% paraformaldehyde in 0.1M phosphate buffer and left for

thirty minutes. The coverslips were washed in phosphate saline buffer and placed in a humidified chamber.

The fixed dorsal root ganglia were cut in sections 10 μm thick using a Bright cryostat and were thaw mounted onto poly-L-lysine coated slides. The slides were labelled and the sections were circled with a PAP pen. This prevents the immuno reagents dispersing to the edge of the slides. The slides were then placed in a humidified chamber at room temperature.

The sections and dissociated cells were blocked with 10% normal donkey serum for one hour. After an hour the excess solution was simply drained off and the primary antibody was applied immediately. Both the sections and dissociated cells were incubated overnight in the combinations of primary antibodies. Table 2.3 shows the primary antibodies used and their dilutions. All antibodies were diluted to the appropriate concentration in antibody buffer (see Appendix 6.1 for composition). After incubation, the sections were washed three times, for ten minutes each time, in phosphate saline buffer. Sections and dissociated cells were then incubated in the appropriate combinations of secondary antibodies diluted in antibody buffer, for one to two hours. Secondary antibodies were raised against the IgG of the species providing the primary antibody and then conjugated to a fluorescent marker.

The sections were washed three times, for ten minutes each time, in phosphate saline buffer. It is important not to allow the sections to dry out at any point. After the final wash the sections were prepared for the microscope. A drop of

mounting solution was placed on the sections and they were covered with a coverslip and sealed with nail polish. The cells plated on the 16 mm coverslips were mounted to a microscope slide and sealed with nail polish. All immunostaining took place at room temperature.

Table 2.3 - The concentrations of the primary and secondary antibodies.

Primary Antibodies	Dilution	Fluorescent Marker	Dilution
N52	1:10,000	AMCA	1:200
IB ₄ (lectin)	1:200	TRITC	1:200
CGRP	1:100	TRITC	1:200
H ₁ receptor	1:100	FITC	1:200

The sources of all antibodies are given in appendix 6.1. The chemical names for the fluorescent markers are given in the abbreviations.

Images were collected using Hamamatsu CCD camera and image Pro Plus software (Media Cybernetics, Silver Spring MD). The fluorescent preparations were examined as soon as possible and stored in the dark at 4°C.

Analysis

There are a number of different ways to quantify immunostaining, however all methods have various drawbacks. In these preliminary experiments the intensity of staining has been judged by eye. For each set of experiments the total number of cells has been counted. A neurone was judged to be positive for a specific stain when there was a clear difference in the intensities of the stained cell from the background. The neurones that stained positive for each antibody were counted. This study also quantified the number of neurones which

expressed more than one marker. This technique does introduce the problem of bias and is limited by the ability of the eye to determine shades of colour.

If this work was continued, the staining would be quantified by the intensity method. Here the intensity of the non-specific staining (background) is determined from a number of sections that are stained with only the secondary antibody. The intensity of the negative cells is counted and the mean background count is obtained. In the remaining sections stained with primary and secondary antibodies, cells are deemed positive if their fluorescent count is greater than two standard deviations above the background count. Problems can occur with this technique even when the secondary antibody is very clean, i.e. there is a very low background count. This situation often leads to an underestimation of the background count and a greater number of cells will appear to be positive. Nevertheless, this technique is a less subjective approach and thus limits bias.

2.4 - Western Blotting

In 1975 Southern introduced a technique which allowed the identification of specific DNA fragments. These fragments had been resolved by gel electrophoresis then transferred to a nitrocellulose membrane. This technique, known as southern blotting, proved to be fundamental to molecular biology. Adaptation of southern blotting, allowed the development of techniques to analyse RNA (northern blotting) and proteins (western blotting). Western blotting uses electrophoresis to separate proteins, which can be immobilised onto a matrix and analysed using various probes including antibodies. The following method describes the use of a gradient SDS-PAGE gel. Electrophoresis in the presence of SDS separates proteins almost exclusively on the basis of mass with smaller polypeptides migrating more rapidly. Gradient SDS page gel provides superior resolution of different bands in the gel. In this study western blotting was used to authenticate the H₁ receptor antibody. A brief overview of the technique is given below but for greater detail see Appendix 6.2.

Protocol

The sample preparation is critical to the running of the gel. The sample proteins need to be solubilised, denatured and disaggregated before loading on the gel. DRG neurones were dissected from male Wistar rats as previously described (see Section 2.1). The DRG neurones were suspended in lysis buffer and homogenised using a clean micropestle. The sample was spun and the

supernatant was removed to a fresh eppendorf where loading buffer was added. The bromophenol blue tracking dye in the loading buffer migrates with the sample allowing the progress of the sample to be monitored during electrophoresis. The two gels were assembled in the gel apparatus and a small volume of running buffer was poured into the chamber between gels with the remaining buffer poured into the external tank. Equal volumes of sample (30 μ l – 50 μ l) were added to each well. Pre-stained molecular weight markers were then loaded into the reference lanes. The gel was run at a constant 100V for 1.5-2 hours.

The gel was then transferred to nitrocellulose membrane at a constant 0.41A for 1.5-2 hours. The transfer apparatus was dismantled and the membrane was placed protein side up in a small tray containing 10% milk solution, covered with cling film and left on a rocker for one hour at room temperature. The milk blocks the non-specific binding sites on the membrane. After an hour the 10 % milk solution was replaced with 5% milk solution containing the H₁R antibody at a dilution of 5 μ g/ml, covered and left on a rocker overnight at 4°C. The membrane was then washed with tris buffered saline tween (TBS-T) buffer then returned to the tray which contained anti rabbit secondary antibody at a dilution of 1:2000 in 5% milk solution, covered and place on a rocker at room temperature for 1-2 hours.

The chemiluminescent agents were mixed together and pipetted onto the blot for two to three minutes. The excess solution was removed and the blot was covered in cling film. In the dark room the blot was exposed to x-ray film for one to two minutes then developed. The bands on the x-ray film were compared with the molecular weight markers on the blot to give an estimation of the molecular weight of protein identified by the bands on the detecting film.

The blot illustrates that the affinity purified anti H₁ receptor antibody recognised a protein band with an apparent mass between 47.4 and 60.7 kDa (see Figure 2.6). This is consistent with previous studies that revealed a molecular mass of 56 KDa for the H₁ receptor protein in rat, mouse and guinea pig (Ruat and Schwartz 1989, Ruat *et al.*, 1988). The other bands that appear on the blot all have an apparent mass greater than that of the H₁ receptor. This suggests that these are complexes e.g. the receptor plus a precursor.

3.0 – RESULTS

3.1 – Dorsal Root Ganglion Morphology

The cell bodies of the sensory afferent fibres lie in the dorsal root ganglia. An electron micrograph (Figure 3.1) of a DRG neuron from a rat has been used

as an example for the morphology of the DRG neurons. The morphology of the DRG neurons is characterized by a large cell body and a long process. The cell body is located in the dorsal root ganglion and the process extends into the dorsal root. The process is composed of two parts: a peripheral process and a central process. The peripheral process is the part of the process that is located in the peripheral nervous system and the central process is the part of the process that is located in the central nervous system.

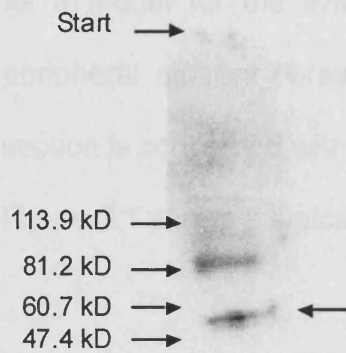


Figure 2.6 – Western blot identifying a band that corresponds to the apparent molecular mass of the H₁ receptor.

The arrow on the right of figure identifies the band which is thought to represent the H₁ receptor.

3.0 – RESULTS

3.1 - Dorsal Root Ganglion Morphology

The cell bodies of the primary afferent fibres lie in the dorsal root ganglia. As discussed earlier (section 1.3.0, p39), DRG neurones have often been used as a model for the investigation of the transduction mechanisms of the peripheral afferent fibres. This approach has been followed here and this section is concerned with the morphological characterisation of these cells.

Figure 3.1 shows a typical field of DRG neurones at day 1 in culture.

Neurones were identified by their appearance and response to a high K^+ stimulus. Under phase contrast optics, healthy viable neurones have a characteristic granular appearance and are surrounded by a bright halo. Satellite cells could be distinguished by their phase dark appearance and the presence of many fine processes. As the DRG neurones were plated at low density it was relatively easy to identify individual neurones. Any cells that did not look healthy or raised doubt as to their neuronal nature (e.g. did not depolarise upon stimulation with high K^+) were not included in this study. Calcium imaging experiments were performed after one to three days *in vitro*. At this time neurite growth from the DRG neurones was limited and there was little overcrowding of non-neuronal cells.

The diameter of the cultured DRG neurones typically ranged from 10 - 30 μm (see Figure 3.2). The majority of neurones had a diameter in the range 12-17.9 μm (63%, $n=118/187$) and the mean diameter was $15.41 \pm 0.27 \mu m$. The culture technique resulted in a high yield of small diameter DRG

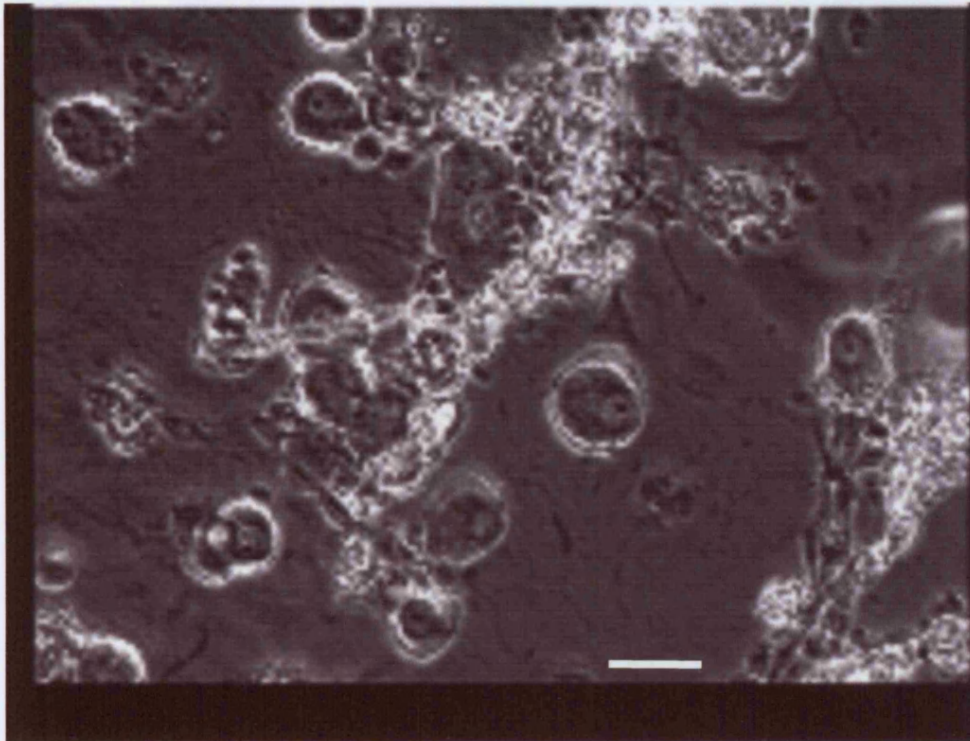


Figure 3.1 - A typical field of cultured DRG neurones at day 1 in culture.

The dorsal root ganglion neurones appear phase dark and are surrounded by a bright halo. The scale bar represents 20 μm .

neurons. Thus, most of the neurons from which recordings were obtained would probably give rise to A δ and C fibres.

3.2 - Sensitivity of Sensory neurones to Histamine

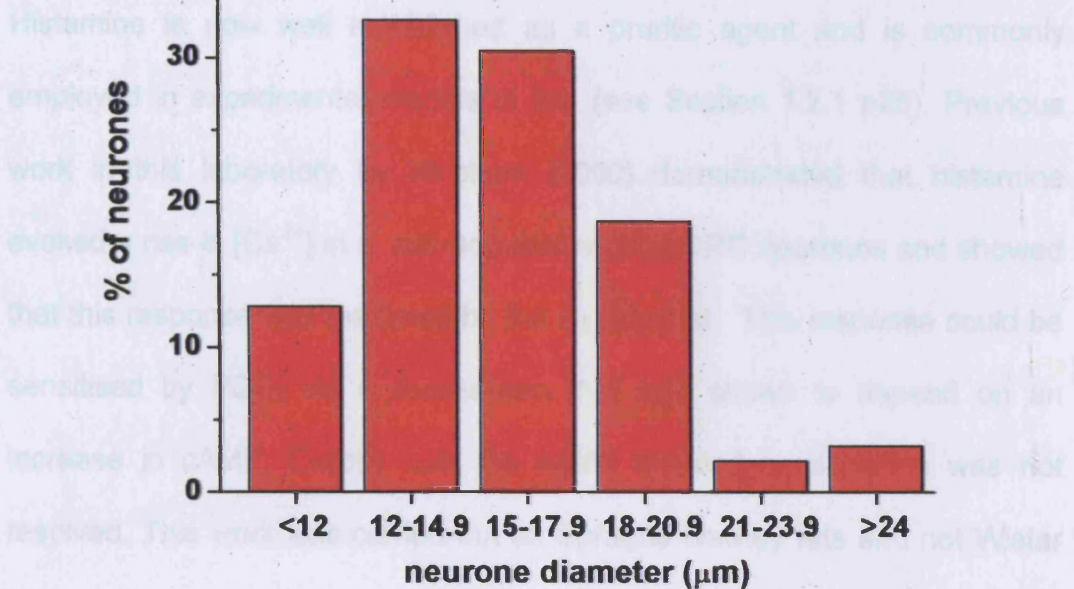


Figure 3.2 - The distribution of neuronal diameters in the sample.

The histogram is derived from a sample of neurones ($n=187$) used for the calcium imaging experiments reported here.

Histamine is a well known vasodilator and a painless irritant. It is commonly employed in experiments involving histamine (see Section 3.2.1 p.23). Previous work by Borsini & Borrelli (1992) demonstrated that histamine evokes a dose-dependent increase in intracellular calcium in rat sensory neurons and showed that this response is mediated by H_1 receptors. The response could be sensitized by pretreatment with H_1 antagonists. The response to an increase in sensory neuron calcium was measured in the present study. The response curve was repeated. The proportion of sensory neurones responding to histamine was 100% in the present study. The difference between the two strains was evident as a slight rightward shift in the dose-response curve (see Figure 3.3). The rightward shift was found not to be statistically significant using Fisher's exact test. Thus, the sensory neurones of Wistar rats are marginally less sensitive to histamine than those of Sprague Dawley rats.

neurones. Thus, most of the neurones from which recordings were obtained would probably give rise to A δ and C fibres.

3.2 – Sensitivity of sensory neurones to histamine.

Histamine is now well established as a pruritic agent and is commonly employed in experimental models of itch (see Section 1.2.1 p25). Previous work in this laboratory by Nicolson (2000) demonstrated that histamine evoked a rise in $[Ca^{2+}]_i$ in a sub-population of rat DRG neurones and showed that this response was mediated by the H₁ receptor. This response could be sensitised by PGE₂ via a mechanism that was shown to depend on an increase in cAMP. Exactly how the cAMP induced sensitisation was not resolved. This work was carried out on Sprague Dawley rats and not Wistar rats as used in these experiments.

Before investigating the mechanism of this sensitisation, the histamine dose response curve was repeated. The proportion of sensory neurones responding to histamine increased from 4% to 32% in Wistar rats and 1% to 35% in Sprague Dawley rats as the concentration rose from 10 μ M to 10 mM histamine. The difference between the two strains was evident as a slight rightward shift in the dose-response curve (see Figure 3.3). The rightward shift was found not to be statistically significant using Fisher's exact test. Thus, the sensory neurones of Wistar rats are marginally less sensitive to histamine than those of Sprague Dawley rats.

As the sensitivity of cells to some agonists (e.g. capsaicin) is known to be affected by time in culture (Bevan and Winter, 1995). The sensitivity of DRG neurones to histamine on successive days was explored. The proportion of cells responding to 10 mM histamine on day 1 (n=48/145) was not significantly different to the number of cells responding on day 2 (n=26/88, p=0.66). Moreover, the proportion of cells responding to 100 μ M histamine on day 1 (30/235) was similar to the number responding on day 2 (n=22/109; p=0.55) and on day 3 (n=11/118; p=0.38). These findings are consistent with those of Nicolson (2000) who showed that the proportion of cells responding to 100 μ M histamine did not change significantly over the course of six days. Thus, the length of time neurones were maintained in culture did not appear to affect their sensitivity to histamine.

Variation in individual sensory neurones responses to histamine

Individual sensory neurones showed considerable variation in the time course and amplitude of the $[Ca^{2+}]_i$ change in response to histamine (see Figure 3.4). Figure 3.4A shows a bright field image of a typical field of DRG neurones. In the field shown, four cells of interest have been labelled. The top three panels of Figure 3.4B show the same field, as pseudocolour images, at various time points during the experiment. Figure 3.4C shows the time course of the individual $[Ca^{2+}]_i$ responses in detail for each cell. At rest, 0 s, the blue colour of the cells illustrated that all four cells had low levels of intracellular calcium. There was very little fluctuation in basal calcium levels of these cells, shown by the straight baselines in Figure 3.4C.

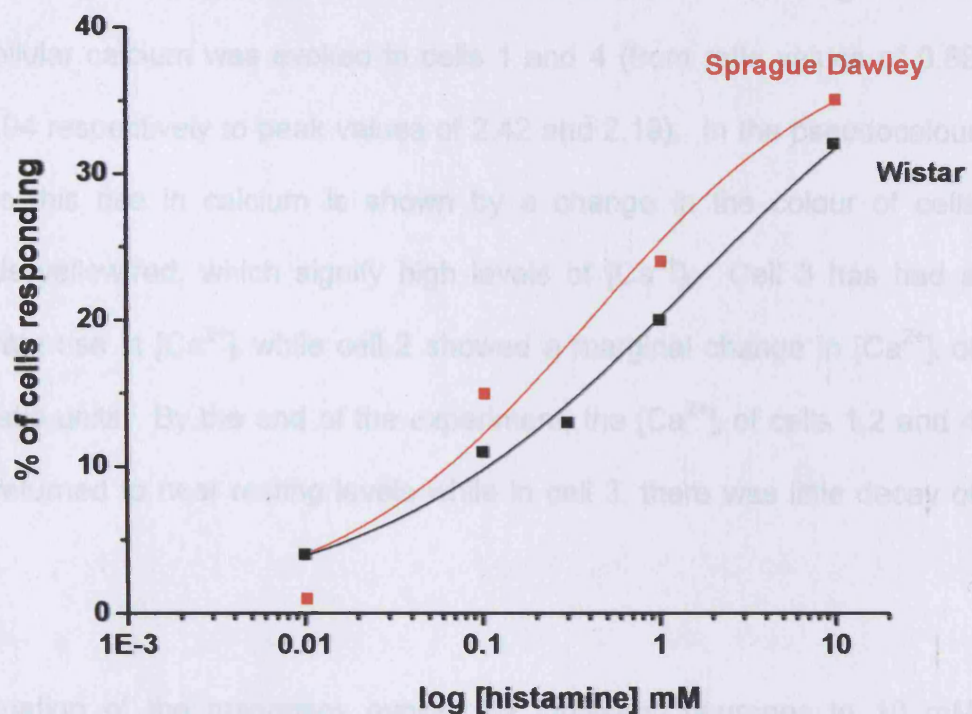


Figure 3.3 – Histamine sensitivity of sensory neurones varies marginally between strains of rat.

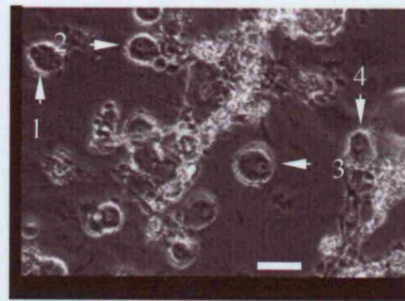
The graph shows the percentage of sensory neurones responding to histamine as a function of concentration. The percentage of neurones responding increases progressively with histamine concentration. The curves were fitted to the Hill equation giving a slope of 0.59 for Sprague Dawley and 0.46 for Wistar. (Sprague Dawley data from Nicolson 2000)

However, upon the addition of 10 mM histamine, at 45 s, a large rise in intracellular calcium was evoked in cells 1 and 4 (from ratio values of 0.88 and 0.94 respectively to peak values of 2.42 and 2.19). In the pseudocolour images this rise in calcium is shown by a change in the colour of cells towards yellow/red, which signify high levels of $[Ca^{2+}]_i$. Cell 3 has had a moderate rise in $[Ca^{2+}]_i$ while cell 2 showed a marginal change in $[Ca^{2+}]_i$ of 0.12 ratio units. By the end of the experiment, the $[Ca^{2+}]_i$ of cells 1, 2 and 4 have returned to near resting levels while in cell 3, there was little decay of $[Ca^{2+}]_i$.

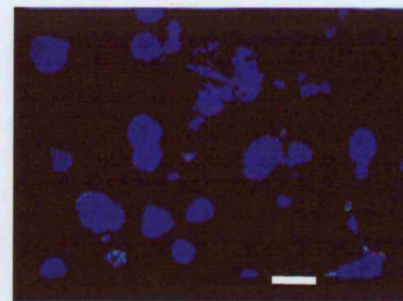
Examination of the responses evoked by individual neurones to 10 mM histamine, showed three distinct patterns (see Figure 3.5A, B and C): In a small number ($n=6/74$) of cells the response was brief and of low amplitude (Figure 3.5A). In around a quarter of the neurones responding to histamine, the rise in intracellular calcium peaked within 20 s, and decayed slowly (Figure 3.5B). However, the majority of cells (50/74) responding to 10 mM histamine showed a response pattern similar to Figure 3.5 C. Upon stimulation there was a large increase in intracellular calcium reaching a peak within 15 s. The response decayed in a biphasic manner with an initial period of rapid decay followed by a slow decline. The profile of the mean response to 10 mM histamine (Figure 3.5 D) was consistent with the large number of responding cells that displayed a response pattern similar to Figure 3.5 C. The proportion of cells showing these individual patterns did not change between day 1 and day 2.

Figure 3.4 – Variation in $[Ca^{2+}]_i$ signal from individual sensory neurones upon stimulation with 10 mM histamine.

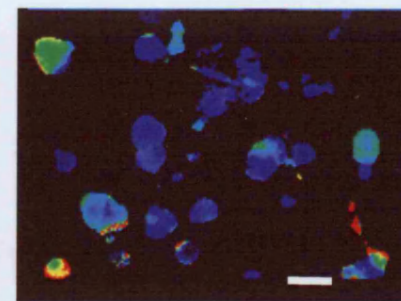
Panel A shows a brightfield image of a typical field of DRG neurones. Four cells have been labelled. Panel B shows the same field of cells, as pseudocolour images, at certain time points during the experiment. Panel C shows the time course of the change in $[Ca^{2+}]_i$ in the four cells upon the addition of 10 mM histamine. The black bar shows the presence of histamine. The scale bar in Panel A and B represents 20 μ m. The spectrum wedge (Panel D) indicates the relationship of the pseudocolour images to the Fura-2 ratio.



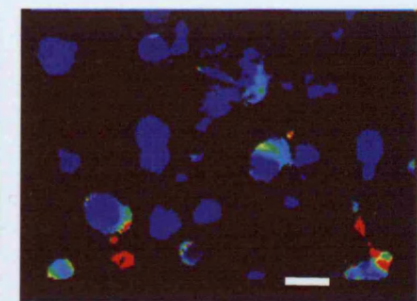
Bright field



B) 0 seconds

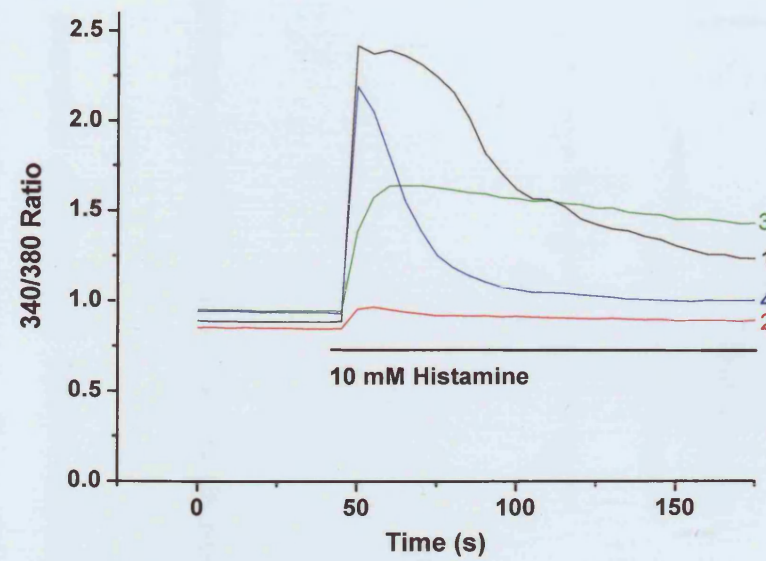


55 seconds

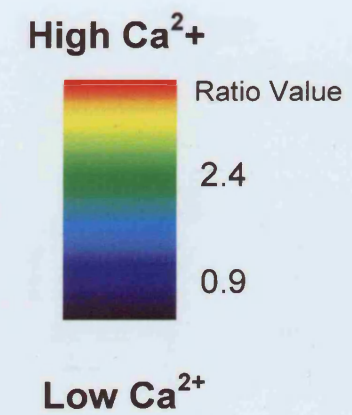


150 seconds

C)



D)



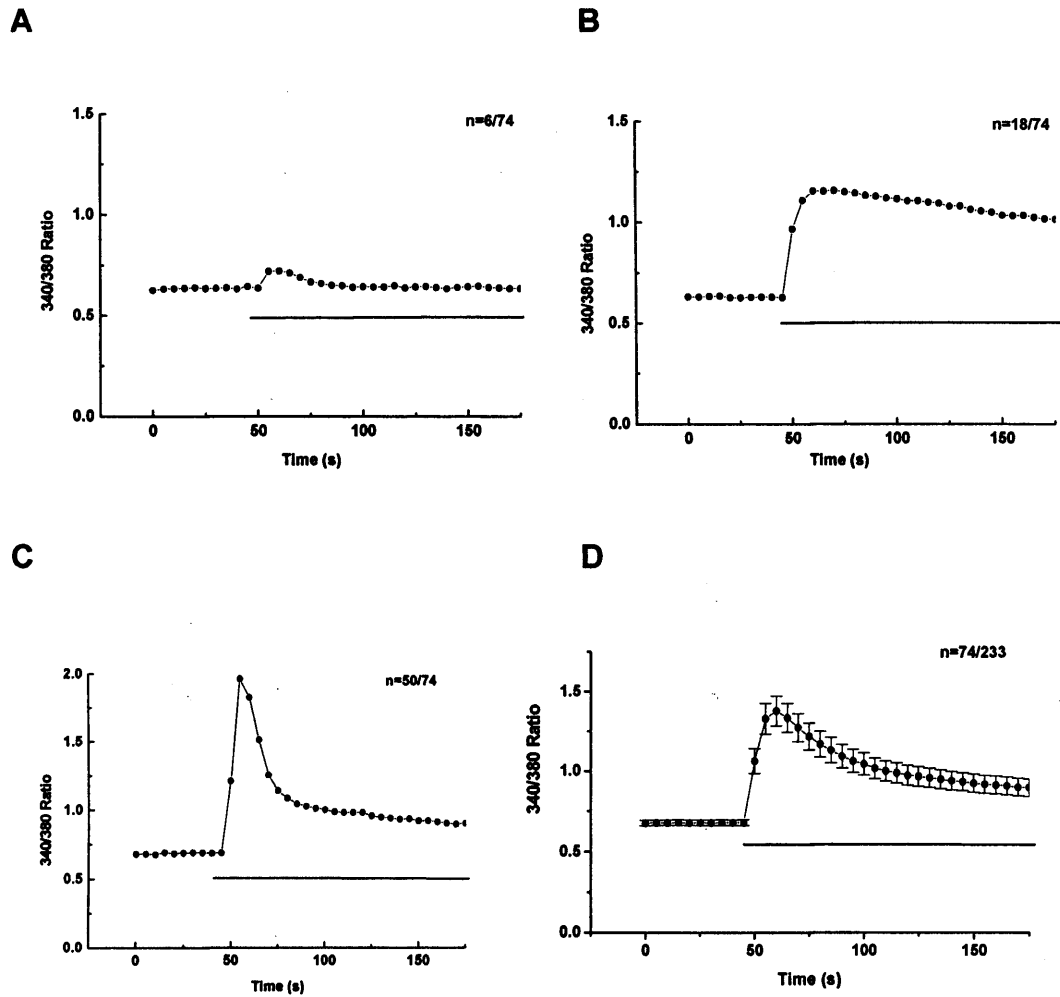


Figure 3.5 – The variation in individual neurones responses to 10 mM Histamine.

Panels A, B and C show typical examples of the three distinct calcium response patterns seen upon stimulation with 10 mM Histamine. The n value gives the number of cells that respond with a similar pattern. Panel D shows the mean calcium response profile for all the responders together with the s.e.m. The black line indicates the presence of histamine.

Effect of concentration on the time course of the $[Ca^{2+}]_i$ response to histamine.

The time course of the mean $[Ca^{2+}]_i$ response varied with the applied histamine concentration. Low histamine concentrations (10 μ M – 1 mM) evoked a rise in $[Ca^{2+}]_i$ that reached a peak within 20 s. The response then very slowly decayed towards baseline (see Figure 3.6 A and B). The application of the highest histamine concentration (10 mM) evoked a rapid rise in 340/380 ratio from a baseline value of 0.67 ± 0.01 to reach a peak value of 1.38 ± 0.09 ($n=74/233$) within 15 s. Following the peak this response then decayed towards baseline, with a time constant of approximately 35 s, (see Figure 3.6C). There was only a marginal change in the time taken to reach a peak with increased histamine concentrations yet the amplitude of the response to histamine more than doubled from 31% to 105% at the highest concentration (see Table 3.1).

Table 3.1 – Time to peak and amplitude of the $[Ca^{2+}]_i$ response to histamine.

Histamine concentrations	Time to Peak (s)	Amplitude %($\Delta R/R$)	n =
100 μ M	20	31	63/563
1 mM	20	23	19/96
10 mM	15	105	74/233

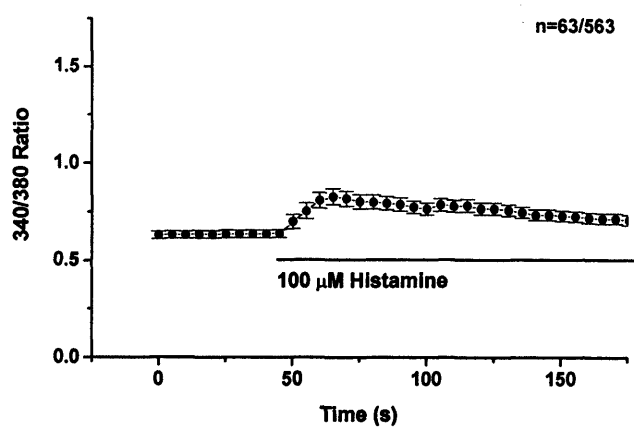
n refers to the number of cells responding to histamine out of the total number of cells tested.

Figure 3.6 - The $[Ca^{2+}]_i$ responses to histamine in sensory neurones.

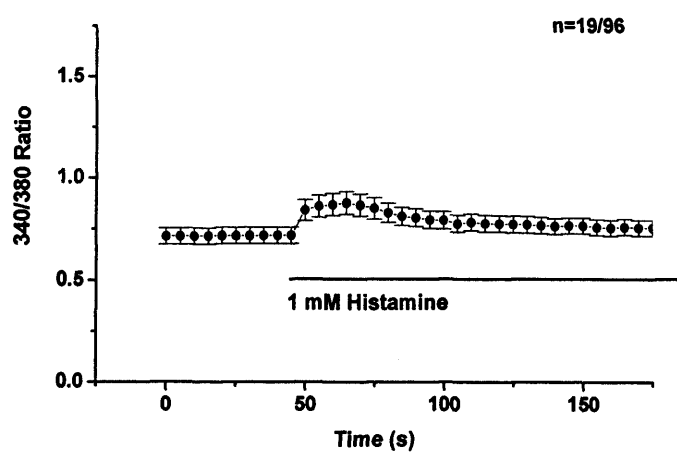
The mean response of histamine sensitive neurones to (A) 100 μ M histamine (B) to 1 mM histamine and (C) to 10 mM histamine.

Histamine was applied at 45 s for the duration of the experiment as indicated by the black bar.

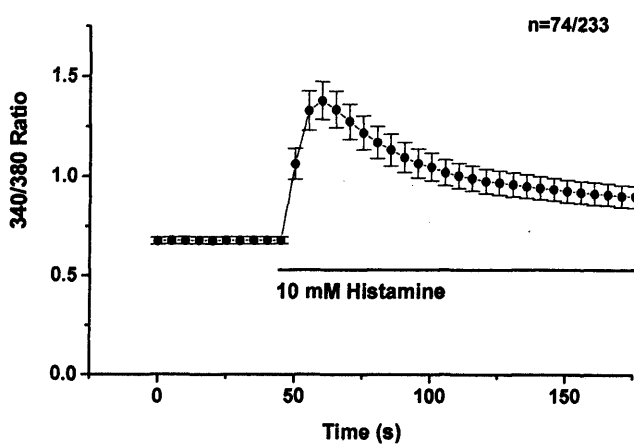
A)



B)



C)



3.3 - PGE₂ induced sensitisation of sensory neurones to histamine.

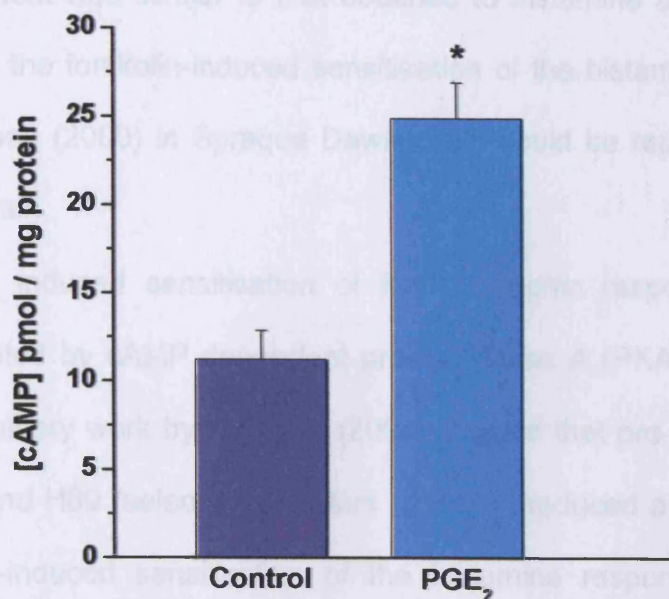
Sensitisation is a key feature of both the itch and pain sensations. Pre-treatment with PGE₂ is known to enhance the sensitivity of sensory neurones to some inflammatory mediators for example bradykinin (Nicol and Cui, 1994, Smith *et al.*, 2000). Furthermore, Nicolson *et al.*, (2002) showed that PGE₂ enhanced the sensitivity of sensory neurones to histamine. This sensitisation was shown to involve cAMP (see Section 4.4, p.167) as Nicolson (2000) also reported a PGE₂ evoked elevation in cAMP in DRG neurones which could be mimicked by forskolin (see Figure 3.7).

Due to the slight difference in the dose response curve for Wistar rats and Sprague Dawley rats it was necessary to test whether this sensitisation could be shown in the Wistar strain. The sensory neurones were pre-treated for 5 minutes with 10 µM forskolin then challenged with 300 µM histamine. The application of forskolin alone did not elicit any response in the neurones and the test concentration of histamine (300 µM) evoked a response in only 13% (n=29/217) of neurones. Five minutes pre-treatment with 10 µM forskolin prior to the histamine challenge significantly increased the proportion of sensory neurones responding to 18% (n=42/230 p= 0.032) (see Figure 3.8).

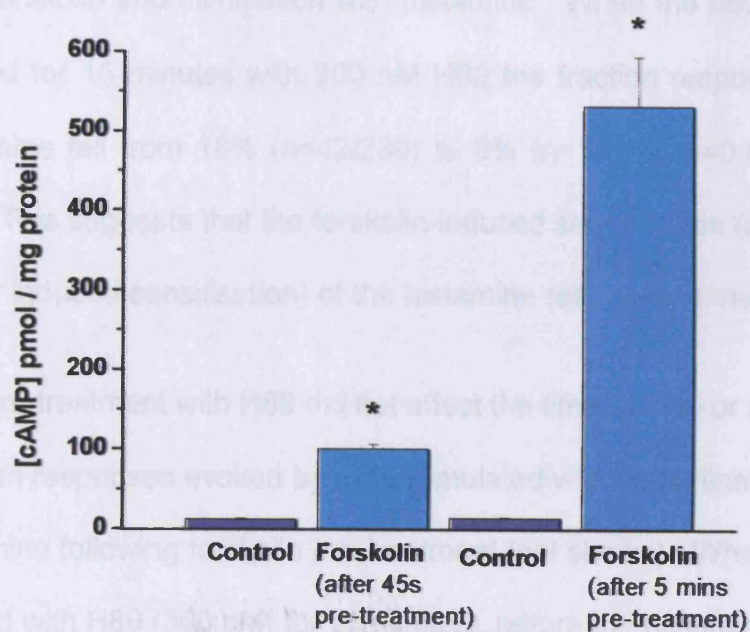
Figure 3.7 - Effect of PGE₂ and forskolin on [cAMP]_i levels in sensory neurones. (Data from Nicolson, 2000).

The cAMP concentration is expressed in relation of the total protein content. The application of (A) PGE₂ and (B) forskolin (for 45 seconds and 5 minutes) produced a significant ($p=0.004$, $p<0.0001$, $p<0.0001$ respectively) increase in intracellular cAMP concentration which is illustrated by the asterisks. Error bars are s.e.m.

A)



B)



Note that the histamine-evoked calcium response following forskolin treatment was similar to that obtained to histamine alone (see Figure 3.9). Thus, the forskolin-induced sensitisation of the histamine response seen by Nicolson (2000) in Sprague Dawley rats could be reproduced in the Wistar rat strain.

PGE₂ induced sensitisation of the bradykinin response is known to be mediated by cAMP dependent protein kinase A (PKA) (Smith *et al.*, 2000). Preliminary work by Nicolson (2000) showed that pre-treatment of cells with PKI and H89 (selective inhibitors of PKA) produced a slight decrease in the PGE₂-induced sensitisation of the histamine response. This raised the possibility that the cAMP induced sensitisation was mediated by PKA. To investigate this, DRG neurones were pre-treated with H89 before treatment with forskolin and stimulation with histamine. When the neurones were pre-treated for 15 minutes with 300 nM H89 the fraction responding to 300 µM histamine fell from 18% (n=42/230) to 8% (n=14/168 p=0.005) (see Figure 3.8). This suggests that the forskolin-induced sensitisation (and therefore the cAMP induced sensitisation) of the histamine response is mediated by PKA.

The pre-treatment with H89 did not affect the time course or amplitude of calcium responses evoked by cells stimulated with histamine alone or with histamine following forskolin pre-treatment (not shown). When cells were treated with H89 (300 nM) for 15 minutes, before the histamine challenge (300 µM), the proportion of neurones showing a rise in $[Ca^{2+}]_i$ was reduced from 13% (n=29/217) to 8% (n=10/130 p=0.166) (see Figure 3.8).

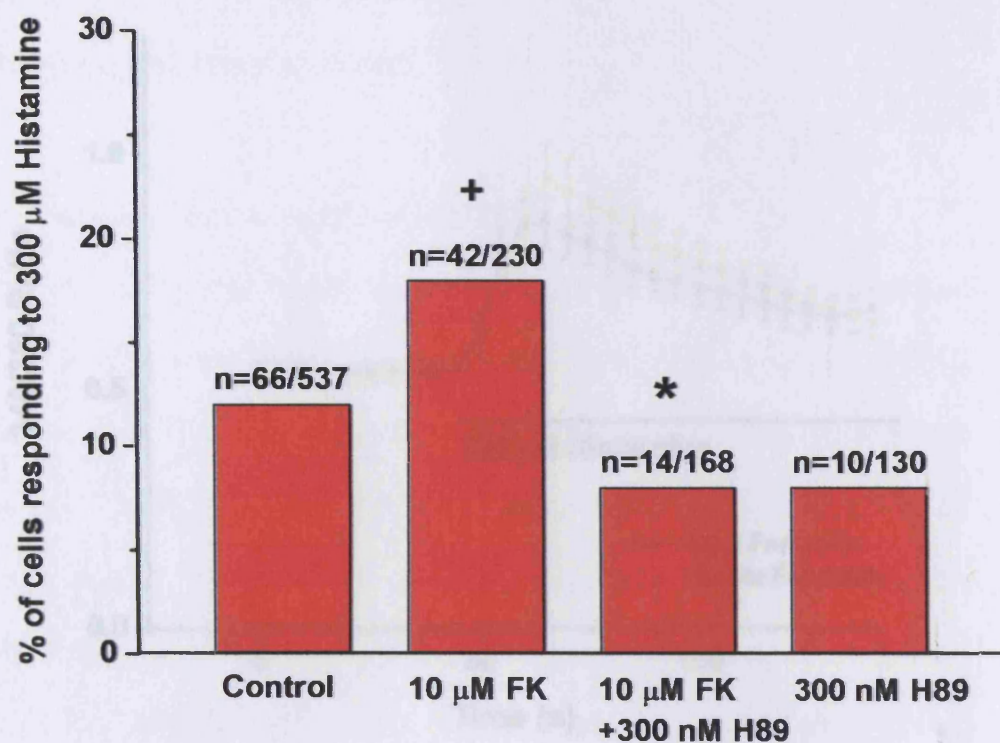


Figure 3.8 – PKA inhibitor, H89, blocks the forskolin-induced sensitisation of the histamine response.

The bar chart shows the percentage of cells responding to 300 μ M histamine after pre-treatment with 10 μ M forskolin, 10 μ M forskolin and 300 nM H89, and 300 nM H89 alone. (+) signifies a significant increase with respect to the control in the proportion of cells responding to histamine after pre-treatment with forskolin ($p=0.032$). The increase observed with the forskolin pre-treatment was significantly reduced ($p=0.005$) when the cells were pre-treated with forskolin and H89 (indicated by asterisk).

3.4 – Chemical and immunological characterisation of the histamine sensitive neurones.

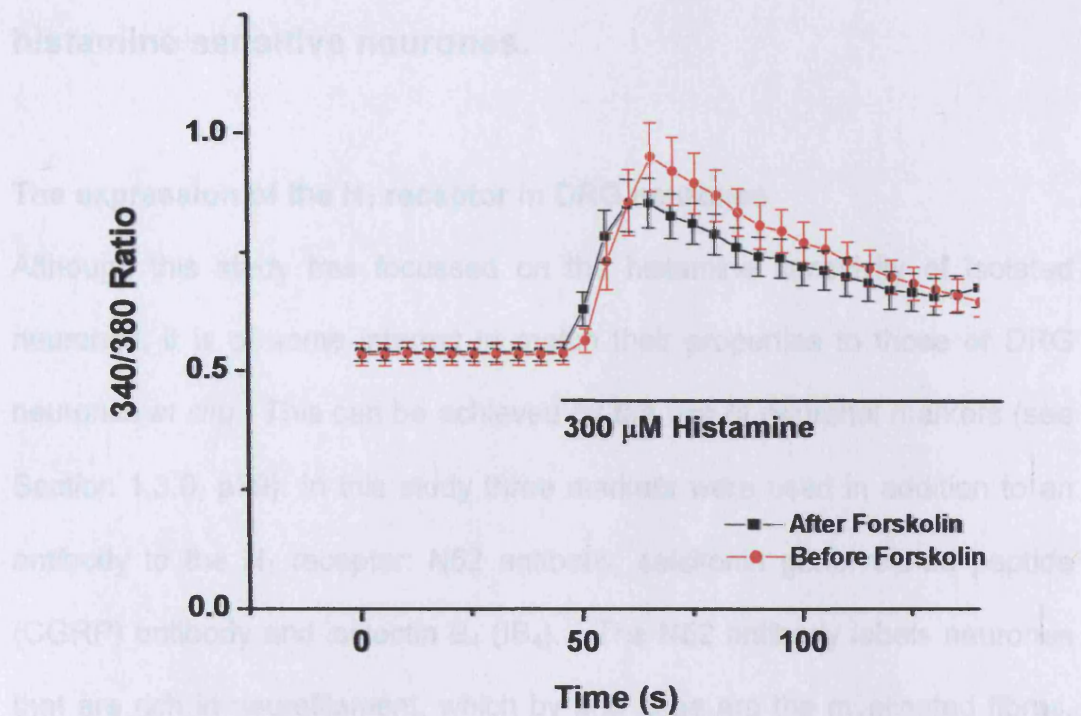


Figure 3.9 – The effect of forskolin on the mean calcium response evoked by neurones upon stimulation with 300 μM Histamine.

The profile of the mean calcium response obtained in histamine sensitive cells after pre-treatment with forskolin is similar to the profile of the mean calcium response obtained to histamine alone. The black line indicates the presence of histamine.

3.4 – Chemical and immunological characterisation of the histamine sensitive neurones.

The expression of the H₁ receptor in DRG neurones

Although this study has focussed on the histamine sensitivity of isolated neurones, it is of some interest to match their properties to those of DRG neurones *in situ*. This can be achieved by the use of neuronal markers (see Section 1.3.0, p39). In this study three markers were used in addition to an antibody to the H₁ receptor: N52 antibody, calcitonin gene related peptide (CGRP) antibody and isolectin B₄ (IB₄). The N52 antibody labels neurones that are rich in neurofilament, which by and large are the myelinated fibres. Thus, this marker will predominantly label DRG neurones that are likely to give rise to myelinated fibres. As discussed in section 1.3.0, the nociceptive primary afferent neurones can be divided into two subgroups, which can be distinguished by CGRP antibody and IB₄ binding. The CGRP antibody identifies neurones that would give rise to peptidergic C fibres, which are NGF responsive. While IB₄ binds to neurones that would give rise to non-peptidergic C fibres, which are GDNF responsive. An H₁ receptor antibody was used to identify the population of DRG neurones expressing the H₁ receptor. Note that the identification of H₁ receptor by the antibody does not necessarily mean that the receptors are functional.

Dissociated and sectioned DRG neurones were triple labelled with anti-H₁ receptor, anti-N52 and either with anti-CGRP or IB₄. Immunolabelled neurones were evenly distributed throughout the cultures and were easily

distinguishable from unlabelled neurones. In control cells, where no primary antibody was applied, no staining was observed. The staining showed a degree of specificity as not all cells were labelled. As mentioned in section 2.3 these are preliminary experiments and thus the observations discussed below are based on the limited data available.

In the dissociated neurones, positive staining for each of the antibodies was seen in a proportion of cells (see Figure 3.10). The merged images and bar chart (Figure 3.10 A, B and D) show that around half of the neurones which are N52 positive also show positive staining for H₁ receptor. Although most small neurones give rise to C fibres some give rise to the thinly myelinated A δ fibres. This pattern of labelling is consistent with previous findings showing that some A δ fibres are histamine sensitive (Handwerker *et al.*, 1987). In Figure 3.10A and D a small fraction of neurones stain with the anti-CGRP antibody which all co-localise with H₁ antibody staining. Moreover, Figure 3.10B and D show that a quarter of the neurones that bind IB₄ co-express the H₁R antibody. Both these markers (CGRP and IB₄) are known to label unmyelinated fibres. Additionally this data suggests that H₁ receptors are expressed on DRG neurones that may give rise to nociceptive fibres. However, in each of the triple labelling experiments there was a small population of neurones that only showed positive staining for the H₁ receptor antibody (see Figure 3.10).

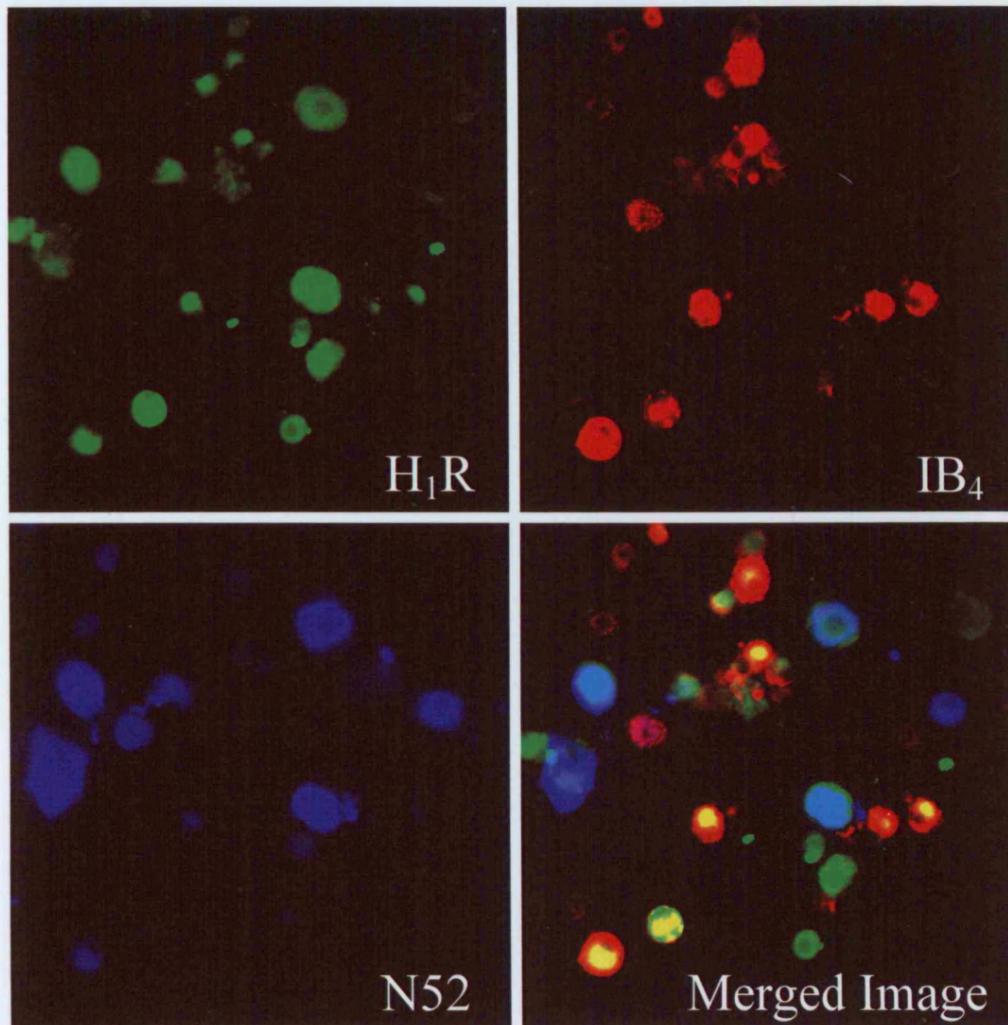


Figure 3.10 – The expression of H₁ receptor in rat DRG neurones.

(A) Dissociated DRG neurones were stained with anti-H₁ receptor antibody, anti-N52 and IB₄. The merged image shows co-localisation of H₁ receptor expressing neurones primarily with IB₄ (neurones in yellow in the merged panel). The merged image also shows neurones with a turquoise colour suggesting that these neurones express both the H₁ receptor and N52.

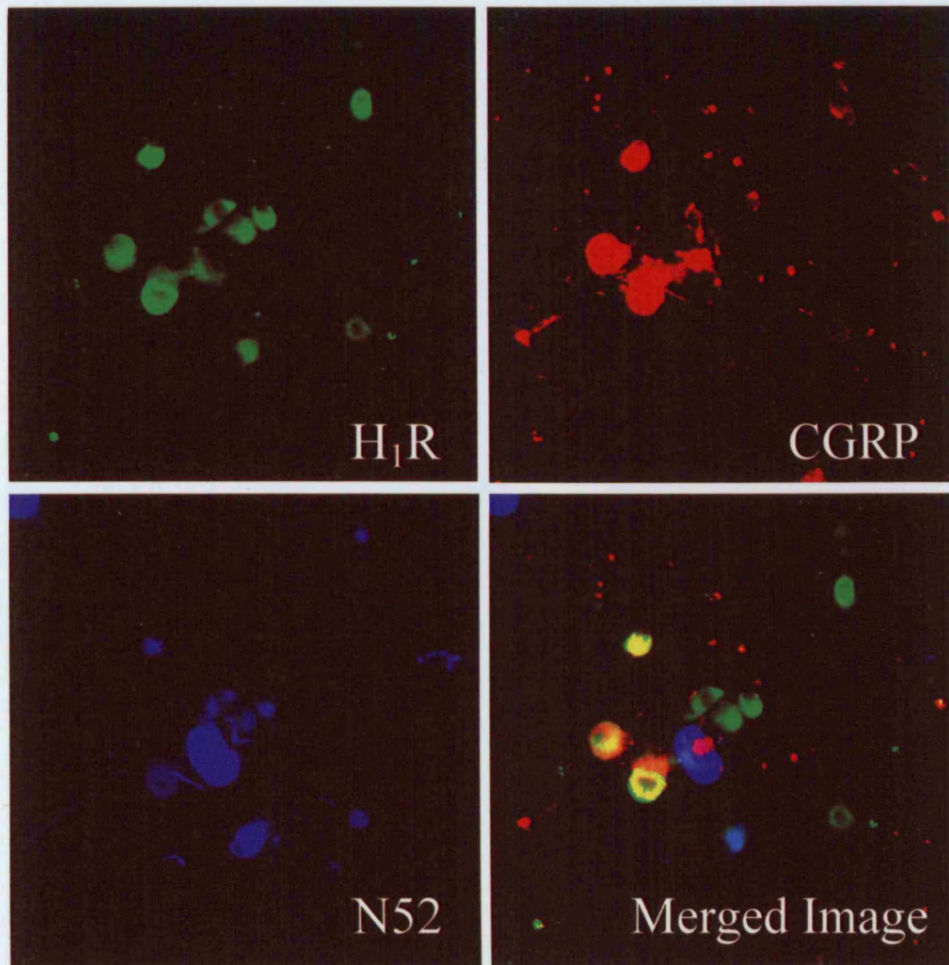


Figure 3.10 B The dissociated neurones were stained for the H₁ receptor, CGRP and N52. The three separate images show that there is positive staining for each of the antibodies. The merge image shows co-localisation of H₁ receptor expressing neurones with CGRP (yellow appearance). The one turquoise cell in the merged image shows the co-localisation of the H₁ receptor expressing neurones with N52.

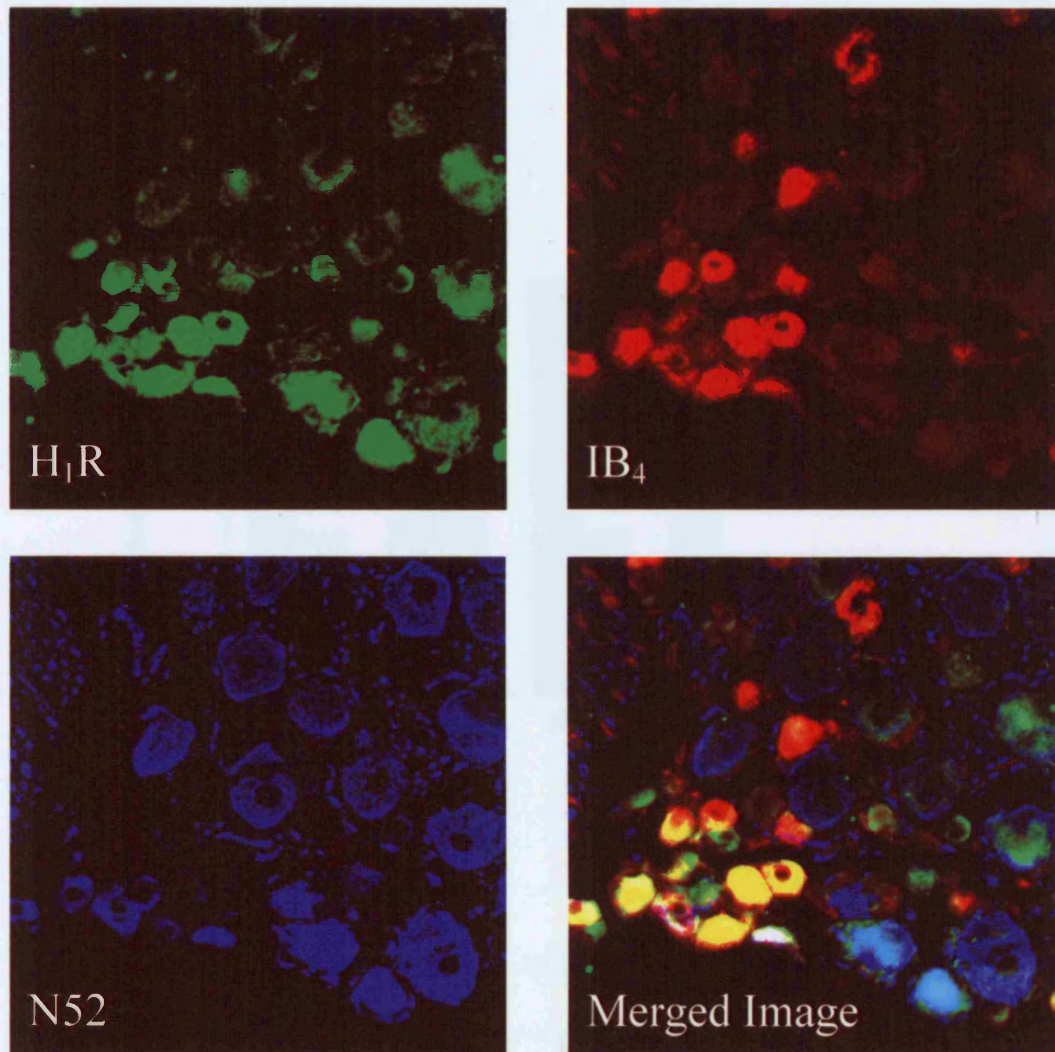


Figure 3.10 C The DRG section has been stained with anti H₁ receptor, IB₄ and anti N52. In the merged image the cells with a yellow appearance show the co-localisation of neurones expressing H₁ receptor and IB₄. The co-localisation of neurones expressing H₁ receptor and N52 is illustrated by the two cells with turquoise appearance.

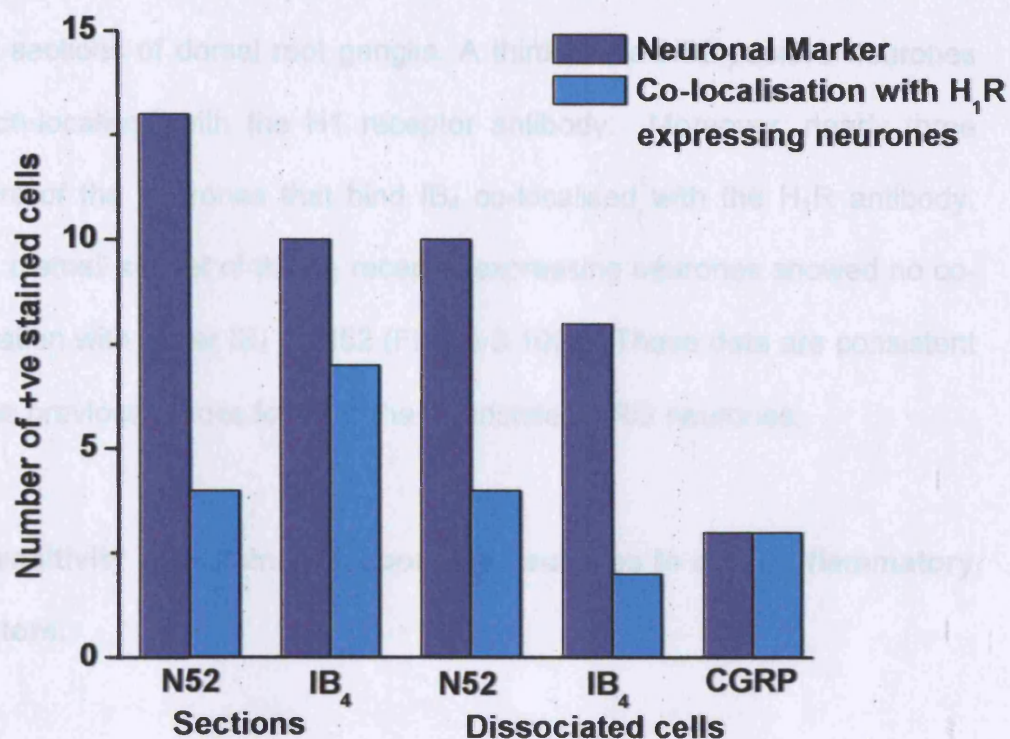


Figure 3.10 D – Co-localisation of the neuronal markers and the H₁R expressing neurones.

H₁ receptor appears to be expressed on neurones that give rise to both unmyelinated and myelinated fibres. Note that these are preliminary experiments with n= 30 for the dissociated cells and for the section. Further experiments are needed to determine the degree of co-expression of the H₁R antibody and each of the neuronal markers.

The triple labelling with anti H₁ receptor, anti N52 and IB₄ was repeated in frozen sections of dorsal root ganglia. A third of the N52 positive neurones were co-localised with the H₁ receptor antibody. Moreover, nearly three quarters of the neurones that bind IB₄ co-localised with the H₁R antibody. Again, a small subset of the H₁ receptor expressing neurones showed no co-localisation with either IB₄ or N52 (Figure 3.10C). These data are consistent with the previous results found in the dissociated DRG neurones.

The sensitivity of histamine responsive neurones to other inflammatory mediators.

As primary afferent nerve endings are exposed to a wide variety of stimuli including pruritic and algogenic agents, it is of interest to investigate whether individual sensory neurones respond to more than one agent. For example, do pruritic sensitive neurones also respond to algogenic agents? However, the responses to histamine, ATP and 5-HT are all mediated by second messenger pathways. Although these agents activate different receptors their signal transduction mechanisms all utilise calcium from internal stores to initiate calcium signals (see later results sections). The release of calcium from the internal stores will deplete them and affect the calcium responses evoked by subsequent stimulation. A previous study showed that DRG neurones which were exposed to a low concentration of histamine (100 μ M), then washed for periods of 5, 30 and 60 minutes and the same field of cells were exposed to a second histamine application resulted in almost all of the neurones not responding to the second application (Nicolson, 2000). For

this reason, only two agonists were tested on the same field of neurones in my investigations. All the agonist concentrations used for the dose response curve were tested on different fields of DRG neurones.

Experiments investigating the proportion of histamine sensitive cells responding to 5-HT (100 μ M) or capsaicin (500 nM) were carried out in Sprague Dawley rats, hence the slightly higher percentage of cells responding.

Of the 11% (n=63/563) of neurones that were histamine sensitive, two thirds (67%) also showed a rise in $[Ca^{2+}]_i$ upon stimulation with 30 μ M ATP. Therefore, a third of the histamine sensitive cells were not responsive to ATP (see Figure 3.11). Just over a fifth (22%) of the histamine responsive cells were shown to respond to 100 μ M 5-HT. Capsaicin (500 nM), the hot component of chillies, evoked a $[Ca^{2+}]_i$ response in nearly half (42%) of the histamine sensitive neurones. This data suggests that there are subpopulations of histamine-sensitive neurones that show different sensitivities to other inflammatory mediators. However, there is also a subpopulation of neurones that is sensitive to histamine and not to the other algogenic agents tested.

Summary of findings

Histamine evokes a rise in $[Ca^{2+}]_i$ in a sub-population of DRG

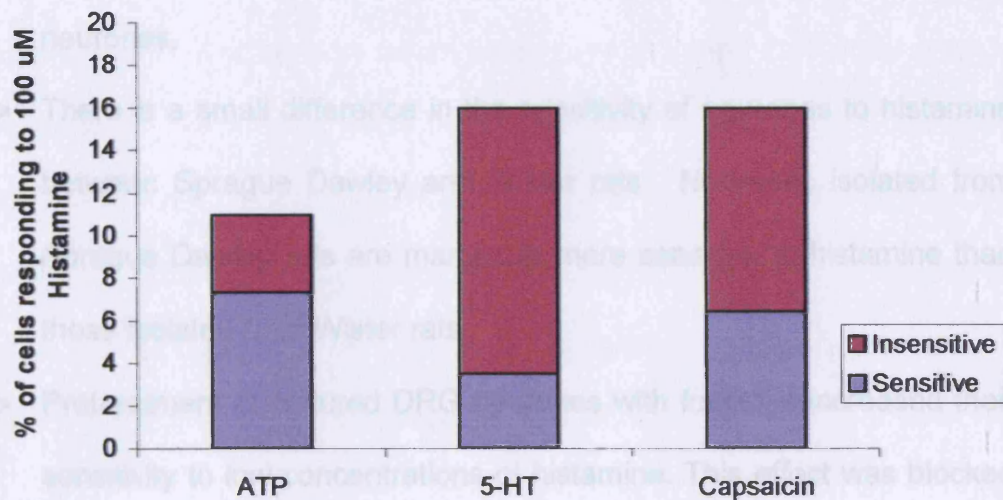


Figure 3.11 – Some histamine-sensitive neurones are sensitive to other inflammatory mediators.

The bar shows the percentage of cells that responded to histamine. The blue segment of the bar illustrates the proportion of these histamine sensitive cells that also responded to a second agonist e.g. ATP (30 μ M) or 5-HT (100 μ M) or capsaicin (500 nM). The purple segment demonstrates the proportion of histamine sensitive cells that did not respond to the second agonists.

Summary of findings

- Histamine evokes a rise in $[Ca^{2+}]_i$ in a sub-population of DRG neurones.
- There is a small difference in the sensitivity of neurones to histamine between Sprague Dawley and Wistar rats. Neurones isolated from Sprague Dawley rats are marginally more sensitive to histamine than those isolated from Wistar rats.
- Pretreatment of cultured DRG neurones with forskolin increased their sensitivity to low concentrations of histamine. This effect was blocked by the PKA inhibitor, H89 suggesting that PKA mediates this sensitisation. Forskolin does not affect the time course or amplitude of the $[Ca^{2+}]_i$ response to histamine.
- A proportion of histamine sensitive neurones are sensitive to other inflammatory mediators.
- Immunostaining showed that the population of neurones expressing H_1 receptor showed some overlap with those stained by anti-neurofilament antibody, CGRP antibody and IB_4 .

3.5 - Characterisation of the calcium response evoked by sensory neurones upon stimulation by 5-HT

Application of 5-HT to sensory neurones evoked a rise in intracellular calcium concentration in a subpopulation of cultured sensory neurones (see Figure 3.12). As the 5-HT concentration was increased from 3 nM to 100 μ M the percentage of cells responding rose from 4% (n=2/53) to 17% (n=56/333). At the highest concentration of 5-HT tested around one fifth of the sensory neurones responded with an increase in intracellular calcium.

As mentioned earlier the length of time cells are maintained in culture can affect their sensitivity to some agonists. On day 1 n= 5/51 cells responded to 100 nM 5-HT which was not significantly different from the n=5/85 (p=0.5) responders on day 2. For cells stimulated with 10 μ M 5-HT the number of responders was n=12/75 on day 1 and n=17/142 on day 2 (p=0.68). The proportion of cells responding to 100 μ M 5-HT on day 1 n=32/210 was not significantly different from the fraction responding on day 2 (n=7/30, p=0.29) and day 3 (n=17/90, p=0.5). It therefore seems that the fraction of cells responding to 5-HT did not change over the first three days in culture.

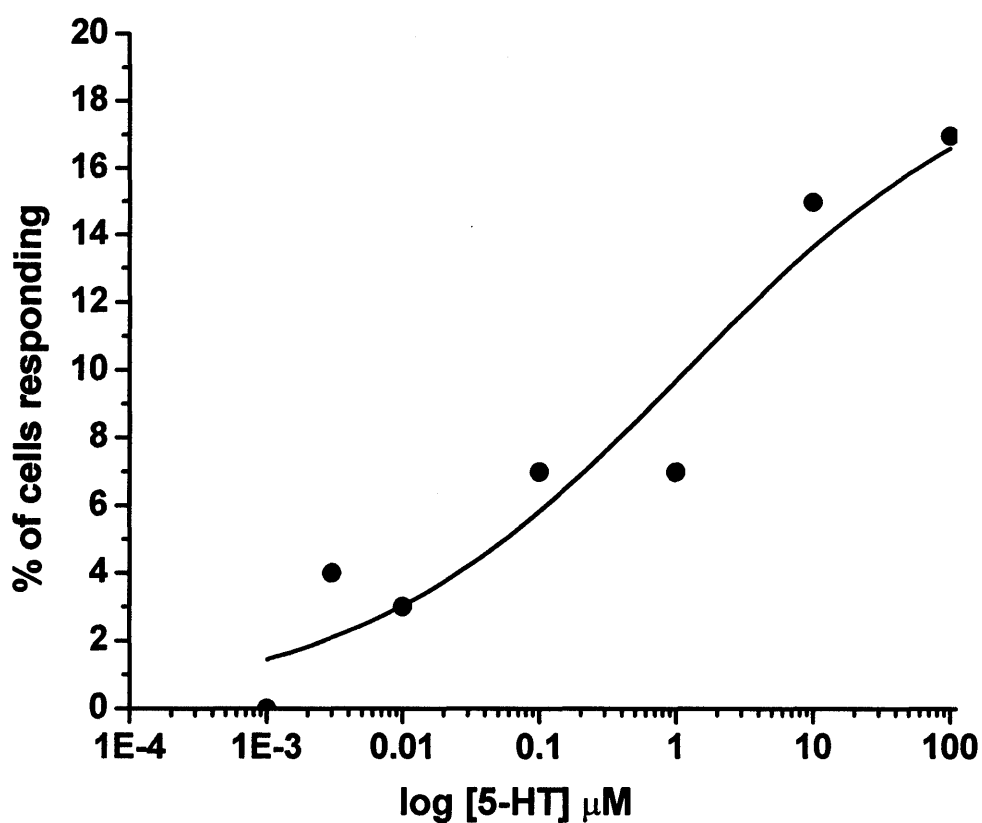


Figure 3.12 – The concentration response curve for the activation of DRG neurones by 5-HT.

The curve is fitted to the Hill equation with a slope of 0.36.

The time course of the calcium responses to 5-HT.

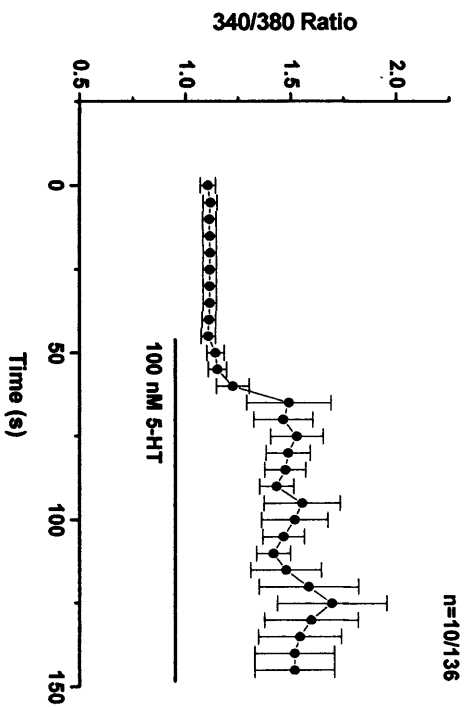
The mean $[Ca^{2+}]_i$ responses to 100 nM, 1 μ M and 100 μ M 5-HT have essentially the same characteristic time course and amplitude (see Figure 3.13). After application of 5-HT, the intracellular calcium rose slowly from baseline, reaching a peak value within 40 seconds. Unlike the responses to ATP and histamine, the intracellular calcium remained elevated for as long as the agonist was present. The responses of individual cells to 100 nM 5-HT are shown in Figure 3.14. Due to the small number of cells that responded to 100 nM 5-HT ($n=10/136$) the variation in amplitude of individual cell responses is reflected in the mean response by an increase in the error bars. The length of time the cells were maintained *in vitro* also did not affect the pattern of the responses observed.

When 100 μ M 5-HT was applied to cells the 340/380 ratio rose from a resting value of 0.86 ± 0.001 to a peak of 1.23 ± 0.06 within 40 seconds (see Figure 3.13C). This increase in ratio corresponds to an increase in intracellular free calcium concentration of approximately 70 nM. Interestingly, the mean amplitude of the responses to 5-HT did not vary with concentration, suggesting that the response is an all or nothing event (see Table 3.2). The time to peak of the mean $[Ca^{2+}]_i$ response to 5-HT was also independent of the concentration of the agonist.

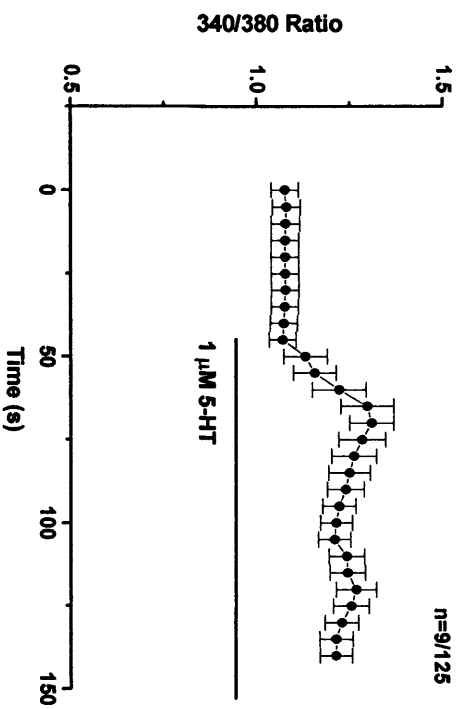
Figure 3.13 - The time course of the $[Ca^{2+}]_i$ response to 5-HT.

The mean $[Ca^{2+}]_i$ response \pm s.e.m. of sensory neurones when challenged with (A) 100 nM 5-HT (B) 1 μ M 5-HT and (C) 100 μ M 5-HT. The black bar indicates the presence of the agonist.

A)



B)



C)

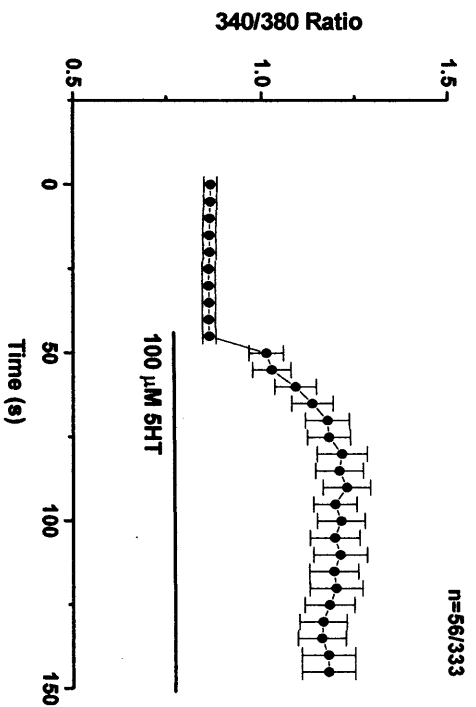


Table 3.2 – Time to peak and amplitude of the responses to 5-HT.

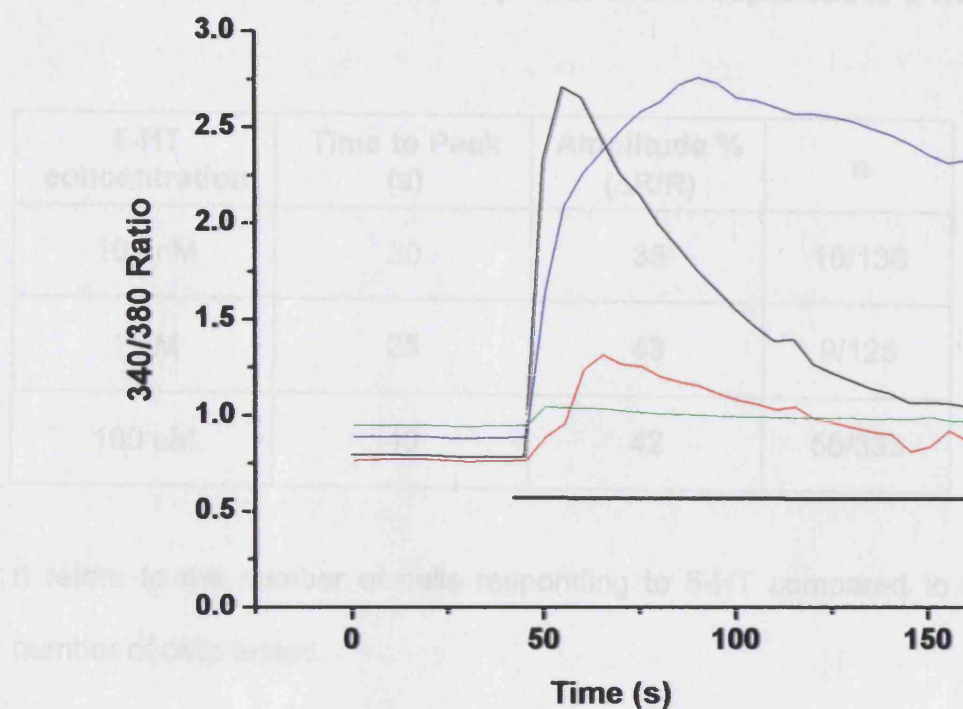


Figure 3.14 – Typical calcium responses evoked by neurones upon stimulation by 100 nM 5-HT.

The black line indicates the presence of 5-HT.

As a subpopulation of sensory neurones respond to 5-HT, we determined the calcium entry mechanism mediating the response to 5-HT. To determine the calcium entry mechanism mediating the response to 5-HT, neurones were challenged with 100 μM 5-HT in a calcium free solution. Removal of extracellular calcium precludes any calcium entry through ionotropic channels, store operated channels and voltage-gated calcium channels. Hence, any observed calcium response to 5-HT would be due to the release of calcium from intracellular stores.

Table 3.2 – Time to peak and amplitude of the responses to 5-HT.

5-HT concentration	Time to Peak (s)	Amplitude % ($\Delta R/R$)	n
100 nM	30	38	10/136
1 μ M	25	43	9/125
100 μ M	40	42	56/333

n refers to the number of cells responding to 5-HT compared to the total number of cells tested.

The calcium entry mechanism/s mediating the response to 5-HT

As the previous section shows, a subpopulation of sensory neurones responded to the application of 5-HT with a rise in $[Ca^{2+}]_i$. Neurones have two sources of calcium to initiate calcium signals; an infinite extracellular source and a finite intracellular source (see section 1.2.5 p.37). To determine the calcium entry mechanism mediating the response to 5-HT, neurones were challenged with 100 μ M 5-HT in a calcium free solution. Removal of extracellular calcium precludes any calcium entry through ionotropic channels, store operated channels and voltage-gated calcium channels. Hence, any observed calcium response to 5-HT would be due to the release of calcium from intracellular stores.

The percentage of cells responding to 100 μ M 5-HT in a calcium-free solution was 18% (n=17/93), which is comparable to the 17% (n=56/333 p=0.76) responding to 100 μ M 5-HT in the 2mM standard calcium solution (see Figure 3.15A). This suggests that calcium released from intracellular stores has a major role in mediating the response of sensory neurones to 5-HT.

To determine whether the calcium response to 5-HT utilises more than one calcium entry mechanism; the time course of the response to 5-HT in a calcium-free solution was examined. The time course and amplitude of the response differed in the presence and absence of external calcium (compare Figure 3.14C and Figure 3.15B). When 5-HT was applied in a Ca^{2+} free medium, the response rose rapidly from a baseline value of 0.82 ± 0.05 to a peak of 1.60 ± 0.25 (n=17/93) within 30 s. After reaching a peak the response was not maintained as shown in the presence of calcium but started to decline slowly towards baseline with a time constant of approximately 33 s. The time to peak was marginally less than the 5-HT response observed in the presence of calcium. However, there was a significant increase (p<0.05) in the amplitude of the response to 5-HT from $41\% \pm 7\%$ in a calcium solution to $95\% \pm 28\%$ in a calcium free solution. This evidence suggests that the initial phase of the response is due to calcium release from internal stores while the maintained phase requires calcium entry via the plasma membrane.

Figure 3.15 - The effect of the removal of calcium on the sensory neurones response to 5-HT.

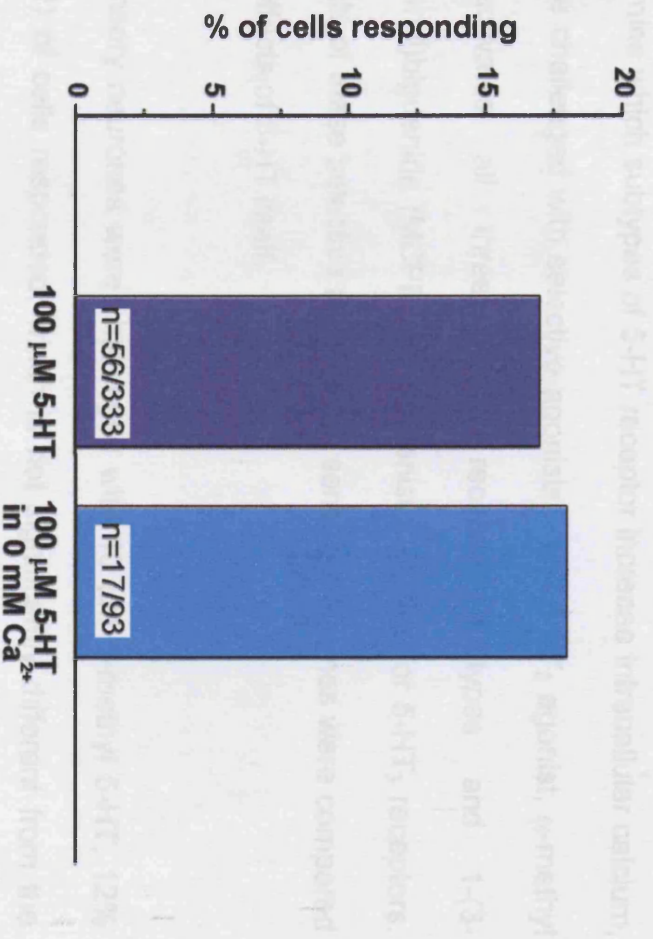
A) Removal of extracellular calcium does not significantly ($p=0.76$) affect the percentage of cells responding to 5-HT.

B) The absence of calcium affects the time course of the $[Ca^{2+}]_i$ response to 5-HT.

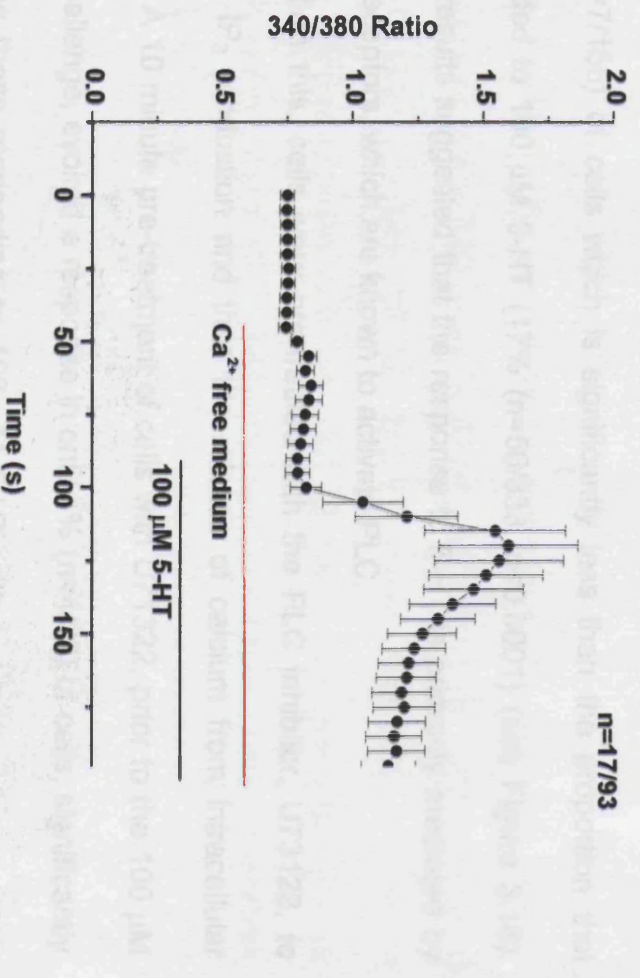
The slight increase in 340/380 ratio at 45 s occurs due to draining and replacement of solutions.

3.5 - The 5-HT receptor subtypes mediating the response to 5-HT in sensory neurones.

A)



B)



3.6 - The 5-HT receptor subtypes mediating the response to 5-HT in sensory neurones.

To determine which subtypes of 5-HT receptor increase intracellular calcium, cells were challenged with selective agonists. The 5-HT₂ agonist, α -methyl 5-HT, activates all three 5-HT₂ receptor subtypes and 1-(3-chlorophenyl)biguanide (MCPB) is an agonist selective for 5-HT₃ receptors. The effects of these selective agonists on sensory neurones were compared with the effects of 5-HT itself.

When sensory neurones were challenged with 10 μ M α -methyl 5-HT, 12% (n=13/112) of cells responded, which is not significantly different from the proportion that responded to 100 μ M 5-HT (17% n=56/333, p=0.227). The 5-HT₃ receptor selective agonist, MCPB (10 μ M), evoked a response in only 4% (n=7/155) of cells which is significantly less than the proportion that responded to 100 μ M 5-HT (17% (n=56/333, p<0.0001) (see Figure 3.16). These results suggested that the response to 5-HT is primarily mediated by 5-HT₂ receptors, which are known to activate PLC.

To confirm this, cells were pre-treated with the PLC inhibitor, U73122, to prevent IP₃ production and thus the release of calcium from intracellular stores. A 10 minute pre-treatment of cells with U73122, prior to the 100 μ M 5-HT challenge, evoked a response in only 4% (n=4/92) of cells, significantly less than those responding to 100 μ M 5-HT with no pre-treatment (17% n=56/333, p<0.0012) (see Figure 3.16). Moreover, this value was not significantly different to the proportion of neurones activated by the 5-HT₃ receptor agonist, MCPB.

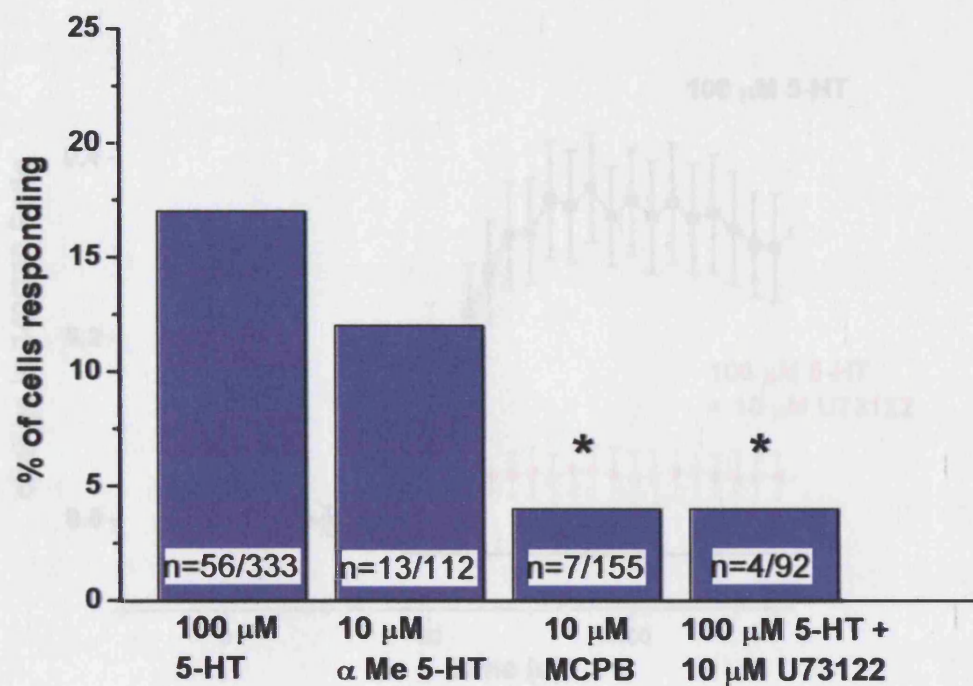


Figure 3.16 – The response to 5-HT is primarily due to activation of 5-HT₂ receptors.

The asterisks indicate a significant decrease in the proportion of cells responding to the 5-HT₃ agonist (MCPB) ($p < 0.0001$), and cells pre-treated with the PLC inhibitor, U73122 ($p < 0.0012$) then challenged with 5-HT in comparison with the 100 μ M 5-HT control. The proportion of cells responding to the 5-HT₂ agonist, α Me 5-HT, is not significantly different from that of 5-HT alone.

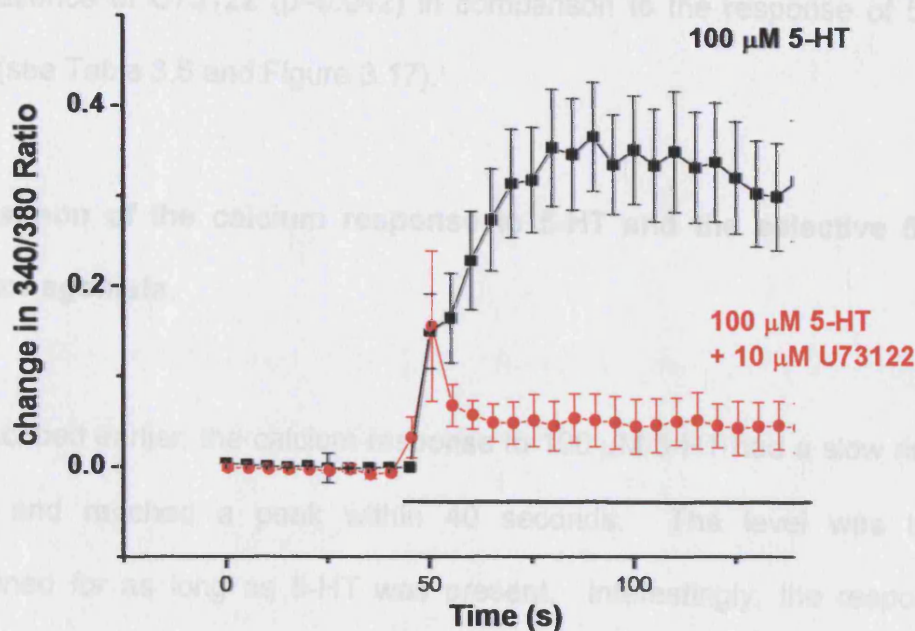


Figure 3.17 - The effect of blocking 5-HT₂ receptors on the [Ca²⁺]_i response evoked to 5-HT.

Blocking the PLC cascade has significantly reduced ($p=0.042$) the amplitude of the [Ca²⁺]_i response to 5-HT. 17% ($n=56/333$) of cells responded to 5-HT however, after pre-treatment with U73122 only 4 % ($n=4/92$) cells responded.

The black line indicates the presence of the agonist.

There was a significant reduction in the amplitude of the response evoked in the presence of U73122 ($p=0.042$) in comparison to the response of 5-HT alone (see Table 3.6 and Figure 3.17).

Comparison of the calcium response to 5-HT and the selective 5-HT receptor agonists.

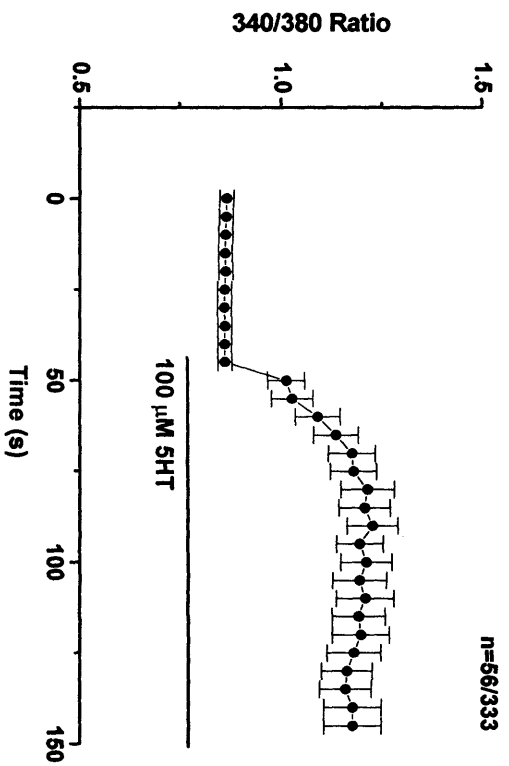
As described earlier, the calcium response to 100 μM 5-HT had a slow rising phase and reached a peak within 40 seconds. The level was then maintained for as long as 5-HT was present. Interestingly, the response characteristics of the 5-HT₂ and 5-HT₃ receptor selective agonists differ from those of the response to 5-HT (see Figure 3.18).

The $[\text{Ca}^{2+}]_i$ response to 10 μM α -methyl 5-HT had a rapid onset rising from a 340/380 ratio of 1.0 ± 0.04 to a peak of 1.94 ± 0.25 ($n=13/112$) within 15 s. The change in 340/380 ratio is estimated to represent an increase in $[\text{Ca}^{2+}]_i$ of 170 nM. The response then showed biphasic decay, a rapid fall for 15 s then a slow decay. The amplitude of the response to the 5-HT₂ agonist was significantly greater ($p=0.035$) than the response to 100 μM 5-HT (see Table 3.3). Interestingly, the amplitude of the response to the 5-HT₂ receptor agonist was comparable to the response of 5-HT in the absence of calcium (see Table 3.3). The calcium response to the 5-HT₃ selective agonist, 10 μM MCPB, had a rapid onset rising from a baseline of 1.10 ± 0.06 to a peak of 1.62 ± 0.09 ($n=7/155$) within 20 s (see Figure 3.18 C). The response then decayed with a time constant of 21 s.

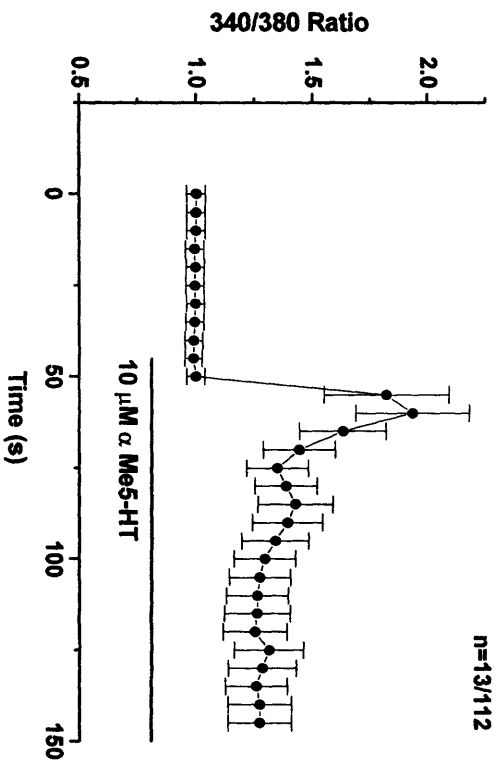
Figure 3.18 - The difference in time courses of the $[Ca^{2+}]_i$ responses evoked by 5-HT and the 5-HT receptor selective agonists.

The figure shows the $[Ca^{2+}]_i$ responses to A) 100 μ M 5-HT, B) 10 μ M α Me5-HT (5-HT₂ agonist) and C) 10 μ M MCPB (5-HT₃ agonist). The black line indicates the presence of the agonist.

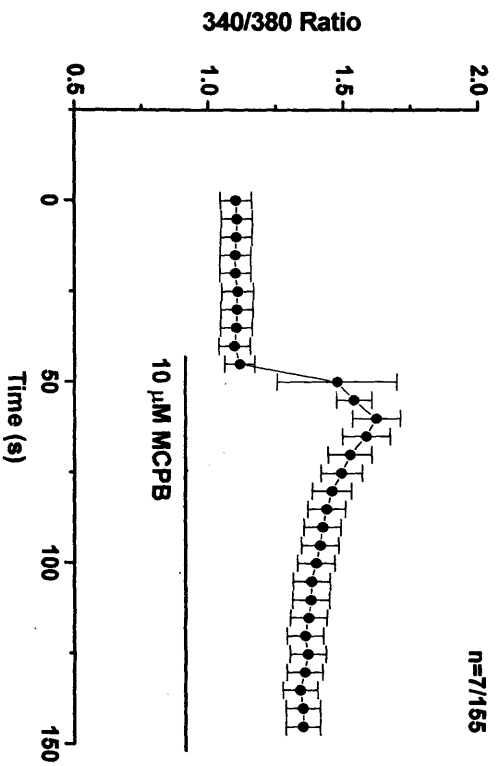
A)



B)



C)



The change in 340/380 ratio upon stimulation with the 5-HT₃ receptor agonist was approximately equivalent to a rise in intracellular calcium of 90 nM. The time to peak of the response was considerably less than that of the response to 100 μ M 5-HT (see Table 3.3).

Table 3.3 - The variation in the characteristics of the calcium responses to 5-HT in different conditions and to the 5-HT receptor selective agonists.

Agonists Concentration	Time to Peak (s)	Amplitude %(ΔR/R)	N	% of cells responding
100 μ M 5-HT	40	41 \pm 8	56/333	17
10 μ M α Me5-HT	15	94* \pm 25	13/112	12
100 μ M 5-HT (Ca ²⁺ free medium)	30	95* \pm 28	17/93	18
100 μ M 5-HT + 10 μ M U73122	15	14* \pm 8	4/92	4
10 μ M MCPB	20	47 \pm 8	7/155	4

The differences in the mean amplitude of calcium responses evoked by cells upon stimulation by a variety of different agonists were compared with respect to the 100 μ M 5-HT control response using the unpaired Welch t test. The asterisk signifies a significant ($p < 0.05$) increase or decrease in the amplitude of the calcium response in comparison to the control.

3.7 – Prostanoid-induced sensitisation of the response to 5-HT.

Pre-treatment with PGE₂ enhances the sensitivity of sensory neurones to histamine (Nicolson, 2000). It is therefore of interest to establish whether this prostanoid can also enhance the sensitivity of sensory neurones to 5-HT.

The sensory neurones were pre-treated for 45 seconds with 1 μ M PGE₂ then challenged with 5-HT. The pre-treatment itself did not elicit any responses but it did enhance the sensitivity of cells to 5-HT. This was demonstrated by a leftward shift of the 5-HT dose-response curve. At lower concentrations of 5-HT (10 nM and 100 nM) there was a significant increase in the percentage of cells responding after PGE₂ pre-treatment (see Table 3.4 and Figure 3.19).

Table 3.4 - PGE₂ pre-treatment enhanced the percentage of cells responding to 5-HT.

5-HT concentration	% of cells responding			
	Before PGE ₂	n	After PGE ₂	n
1 nM	0	0/84	3	1/36
3 nM	4	2/53	11	7/66
10 nM	3	2/63	15*	11/75
100 nM	7	10/136	18*	27/147
1 μ M	7	9/125	15	16/106
10 μ M	15	29/199	19	16/83

The asterisks indicate a significant increase ($p < 0.05$) in the percentage of cells responding to 5-HT after PGE₂ pre-treatment in comparison to 5-HT alone using Fisher's exact test.

The sensitisation of the response to 5-HT produced by PGE₂ is consistent with a reduction in response threshold. Furthermore, as Figure 3.19B shows, pre-treatment with PGE₂ had no significant effect on the time course or amplitude of the [Ca²⁺]_i response evoked by 5-HT. The [Ca²⁺]_i response to 100 nM 5-HT after 1 µM PGE₂ pre-treatment had a slow rising onset reaching a peak in 25 s. Similar to the control cells, the evoked response was sustained with no decay for as long as 5-HT was present. To determine whether cAMP played a role in the PGE₂ induced sensitisation of the 5-HT response the effects of forskolin were investigated. Treatment of sensory neurones with forskolin is known to increase cAMP levels (Nicolson 2000, see Figure 3.7). When sensory neurones were pre-treated for 5 minutes with 10 µM forskolin the number of cells responding to 100 nM 5-HT significantly increased from 7% (n=10/136) to 18% (n=11/60 p=0.0419) (see Figure 3.20). This suggests a role for cAMP in the PGE₂ induced sensitisation of the response to 5-HT.

To determine if PKA had a role in the prostanoid-induced sensitisation of the response to 5-HT, cells were pre-treated for 15 minutes with 300 nM H89, a PKA inhibitor, prior to 1 µM PGE₂ pre-treatment. After PGE₂ pre-treatment 18% (n=27/147) of neurones responded to 100 nM 5-HT while after H89 and PGE₂ pre-treatment only 8% (n=7/89 p=0.03) of cells responded to 100 nM 5-HT (see Figure 3.20). This result is consistent with the involvement of PKA in the prostanoid induced sensitisation of the 5-HT response.

Figure 3.19 - PGE₂ pre-treatment enhanced the sensitivity of cells to 5-HT.

A) The log dose response curve for 5-HT alone (black line) and for 5-HT after pre-treatment with PGE₂ (red line).

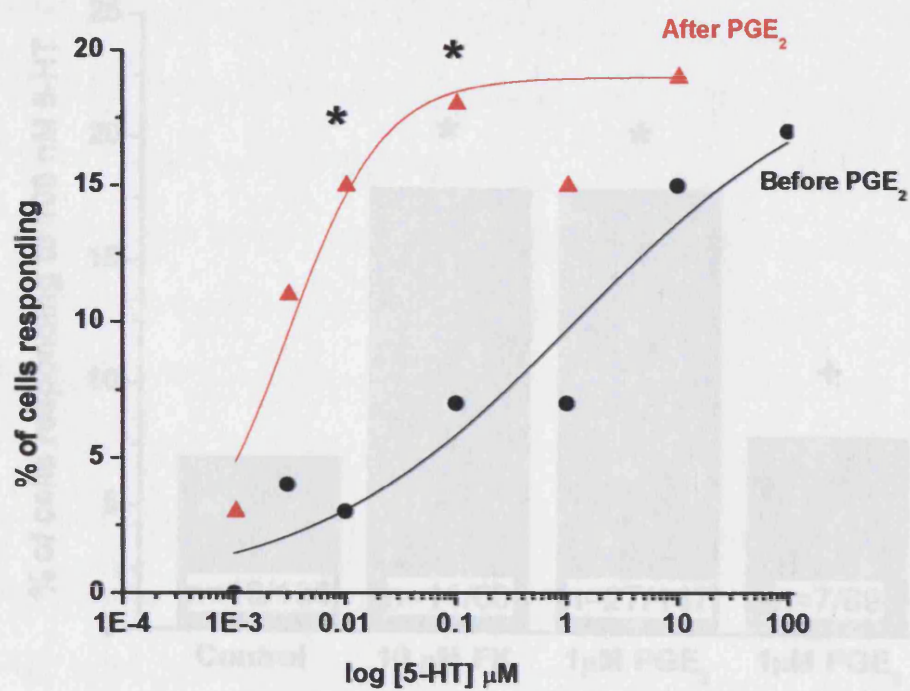
PGE₂-induced sensitisation has significantly (illustrated by asterisks) increased the percentage of sensory neurones responding to lower concentrations of 5-HT.

The curves are fitted to the Hill equation giving a slope of 0.36 for 5-HT alone and 1.0 for 5-HT after PGE₂ pre-treatment.

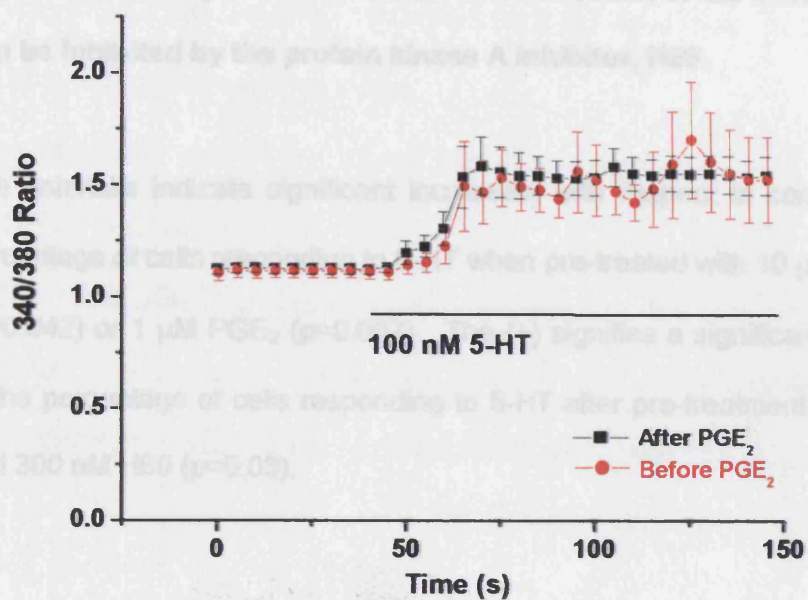
B) The time course and amplitude of the [Ca²⁺]_i response to 5-HT was not affected by prior PGE₂ pre-treatment.

PGE₂ also had no effect on the basal level of [Ca²⁺]_i.

A)



B)



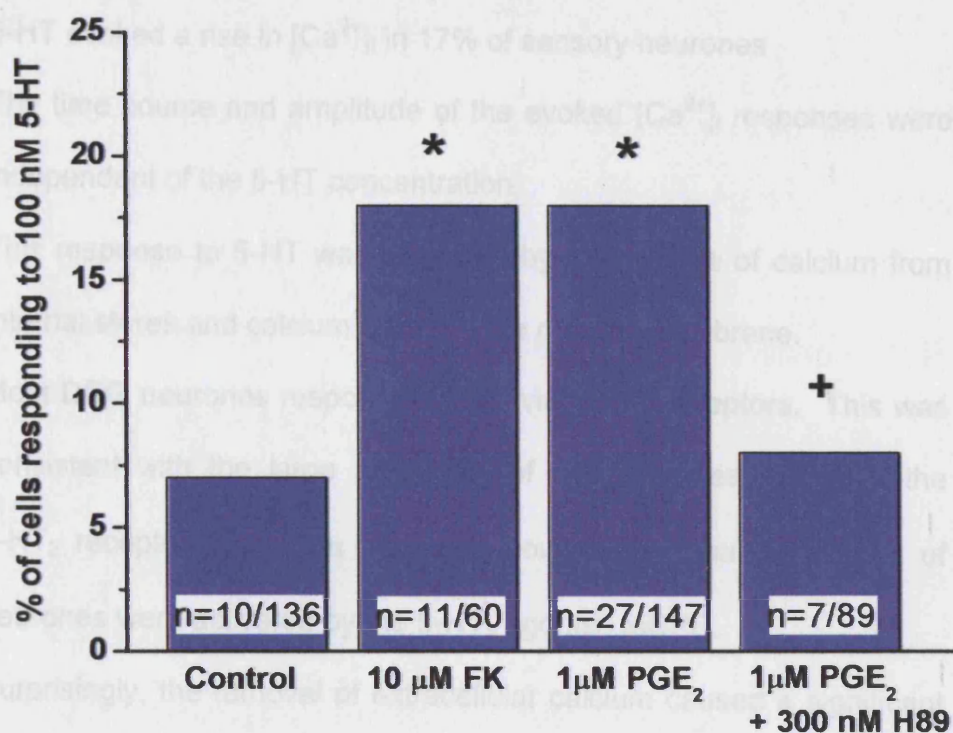


Figure 3.20 - The prostanoid induced sensitisation of the 5-HT response can be inhibited by the protein kinase A inhibitor, H89.

The asterisks indicate significant increases, with respect to control, in the percentage of cells responding to 5-HT when pre-treated with 10 μ M forskolin ($p=0.042$) or 1 μ M PGE₂ ($p=0.007$). The (+) signifies a significant decrease in the percentage of cells responding to 5-HT after pre-treatment with PGE₂ and 300 nM H89 ($p=0.03$).

Summary of findings

- 5-HT evoked a rise in $[Ca^{2+}]_i$ in 17% of sensory neurones
- The time course and amplitude of the evoked $[Ca^{2+}]_i$ responses were independent of the 5-HT concentration.
- This response to 5-HT was mediated by the release of calcium from internal stores and calcium entry via the plasma membrane.
- Most DRG neurones respond to 5-HT via 5-HT₂ receptors. This was consistent with the large proportion of cells that responded to the 5-HT₂ receptor agonist, α Me 5-HT; however, a small proportion of neurones were activated by the 5-HT₃ agonist, MCPB.
- Surprisingly, the removal of extracellular calcium caused a significant increase in the amplitude of the response to 5-HT which was comparable to the response of the 5-HT₂ agonist, α Me5-HT.
- PGE₂ sensitised the DRG neurones to 5-HT as shown by a leftward shift in the dose response curve.
- Pre-treatment with PGE₂ did not alter the amplitude or time course of the $[Ca^{2+}]_i$ response.
- PGE₂ sensitisation of the sensory neurones to 5-HT was mimicked by forskolin and blocked by the PKA inhibitor, H89. This implies that the sensitisation results from a phosphorylation reaction mediated by PKA.

3.8 - The sensitivity of sensory neurones to ATP

Application of ATP evoked a rise in intracellular calcium concentration in a large fraction of isolated sensory neurones. The percentage of cells responding rose sharply from 14% (n=15/108) at 1 μ M ATP to 69% (n=74/107) at 10 μ M ATP. The percentage of cells responding then appeared to plateau between 10 μ M and 100 μ M ATP (see Figure 3.21).

In common with the responses to histamine and 5-HT the fraction of DRG neurones responding to ATP did not change during the first three days in culture. On day 1 n=4/33 cells responded to 1 μ M ATP which is comparable to the n=4/30 (p=1.0) responding on day 2 and n=7/45 (p=0.75) on day 3. At the highest concentration, 100 μ M ATP, there was no difference in percentage of cells responding on day 1 (n=19/24) compared to day 2 (n=49/69, p=0.6). Thus, the sensitivity of cells to each concentration of ATP tested was not affected by time in culture.

Variation of individual sensory neurones responses to ATP.

Figure 3.22 demonstrates that individual sensory neurones can produce very different $[Ca^{2+}]_i$ signals in response to the same concentration of agonist, in this case 100 μ M ATP. Panel A shows a bright field image of a typical field of DRG neurones, in which four cells of interest have been labelled. Panel B shows the same field of cells as pseudocolour images. The lower panel (C)

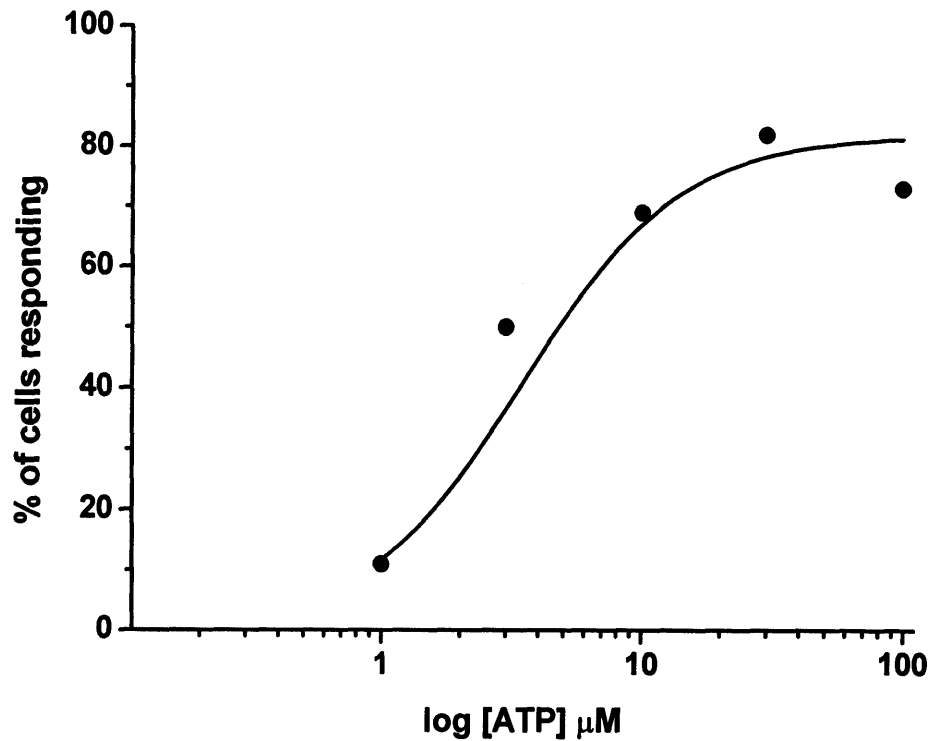


Figure 3.21 - ATP evoked a rise in $[\text{Ca}^{2+}]_i$ in a large proportion of sensory neurones.

The graph shows the number of cells responding as a percentage of the total number of cells tested at each ATP concentration (1 μM to 100 μM).

The curve is fitted to the Hill equation with a slope of 1.42.

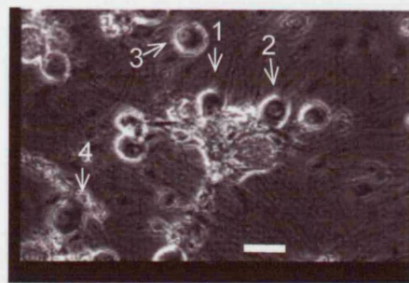
shows how the time course and amplitude of the responses differed between individual cells. Initially all of these cells had low levels of intracellular calcium shown by the blue colour of the cells (Panel B). After 45 s 100 μM ATP was added. After the addition of ATP, all four cells showed a rise in intracellular calcium. Cell 1, 3 and 4 showed moderate increase while the red colour of cell 2 (in Panel B) signifies a large rise in intracellular calcium. Cell 2 rose from a baseline of 1.04 to 1.17 initially, then a second increase occurred reaching a peak of 4.8 (see Figure 3.22C). The response then rapidly decayed towards baseline. Cells 1 and 3 have comparatively weak responses to ATP. Upon reaching a peak, the $[\text{Ca}^{2+}]_i$ in all cells decay towards baseline. All four cells responded to the subsequent application of 50 mM K^+ (K^+ depolarisation) with a marked rise in intracellular calcium.

Characterisation of the mean $[\text{Ca}^{2+}]_i$ responses to increasing concentrations of ATP.

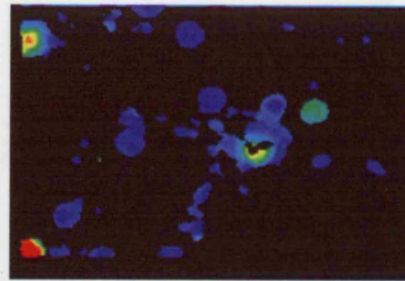
The mean response of sensory neurones to 1 μM ATP rose from a baseline value of 1.05 ± 0.02 to a peak of 1.23 ± 0.06 over 40 s. This ratio change corresponds approximately to a rise of 40 nM free $[\text{Ca}^{2+}]_i$. Thereafter, $[\text{Ca}^{2+}]_i$ decayed towards baseline (see Figure 3.23A). The $[\text{Ca}^{2+}]_i$ response to 10 μM ATP reached its peak value within 20 s which was considerably faster than the response to 1 μM ATP (see Table 3.5). The change in ratio value of 0.5 would give an estimated 90 nM rise in $[\text{Ca}^{2+}]_i$. After reaching the peak the calcium response slowly decayed towards baseline (see Figure 3.23B).

Figure 3.22 – The variation in $[Ca^{2+}]_i$ responses to ATP between sensory neurones

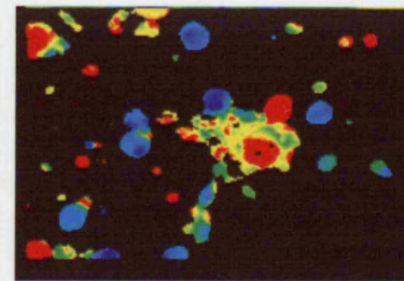
Panel A shows a field of DRG neurones in which selected cells of interest have been labelled. Panel B shows pseudocolour images of the same field at progressive times. They illustrate the change in intracellular calcium concentration of cells upon the addition of ATP and 50 mM K^+ . Panel C shows the time courses in more detail for the individual neuronal responses to the addition of ATP at 45 s and high K^+ at 150 s. The spectrum wedge (panel D) relates the colour change of the cells to the change in 340/380 ratio. The scale bar in panel A represents 20 μm .



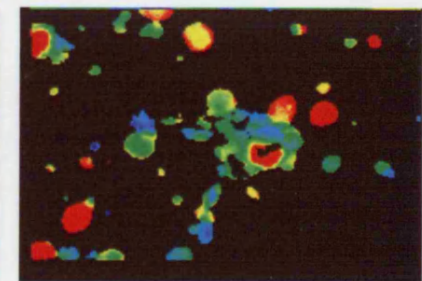
A Bright field



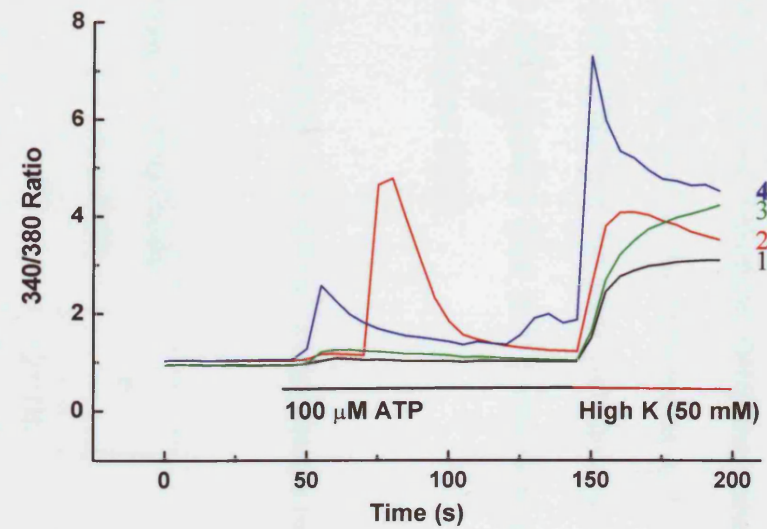
B 0 seconds



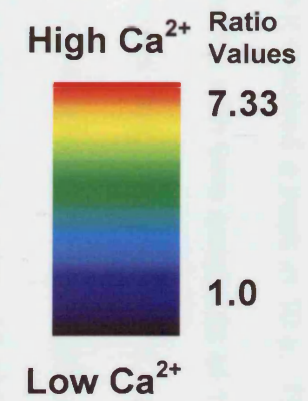
80 seconds



170 seconds



C



D

The $[Ca^{2+}]_i$ response to 100 μ M ATP had a similar time course to the responses to lower concentrations but reached a peak in 10 s. The response showed biphasic decay towards baseline with time constants of 15 s and 127 s (see Figure 3.23C). The ratio changed from a baseline of 1.1 ± 0.02 to a peak value of 2.19 ± 0.17 after stimulation with 100 μ M ATP. This corresponds to an increase in $[Ca^{2+}]_i$ from approximately 10 nM to 230 nM. Thus, the time to peak and amplitude of the ATP response were dependent on concentration (see Table 3.5).

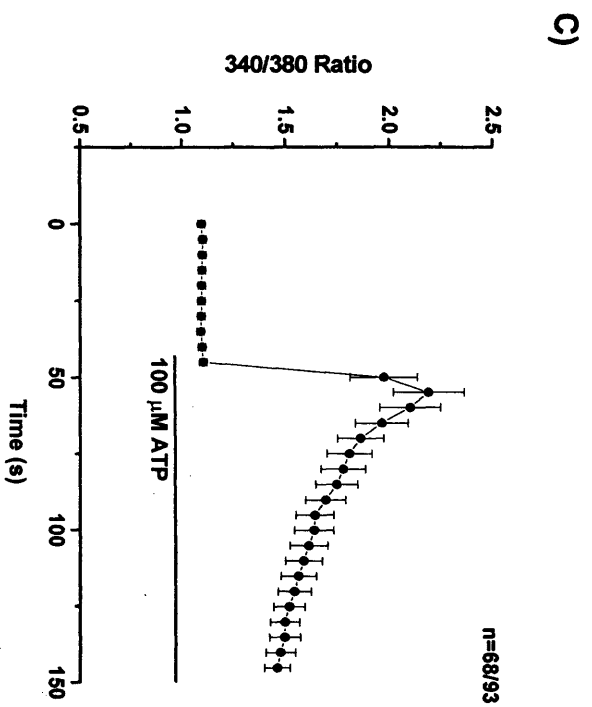
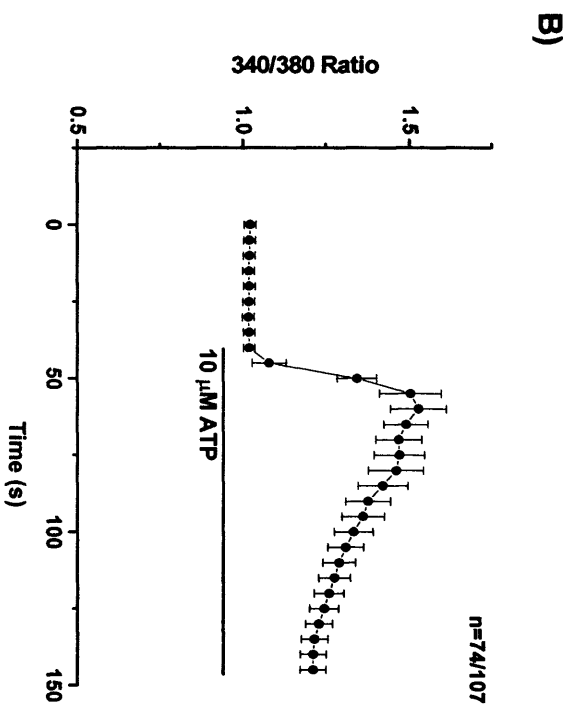
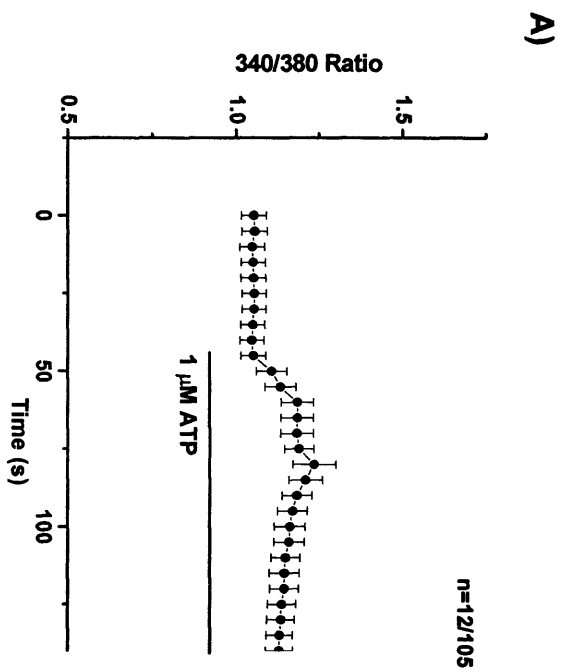
The patterns of the responses to ATP in individual DRG neurones were essentially the same (see Figure 3.24). As for the responses to 5-HT only the amplitude varied between cells. They did not vary with time in culture. Prolonged exposure to 30 μ M ATP showed that $[Ca^{2+}]_i$ did not return to baseline for more than 200 s (see Figure 3.25).

Table 3.5 – Time to peak and amplitude of the $[Ca^{2+}]_i$ responses to ATP.

ATP concentrations	Time to Peak (s)	Amplitude % ($\Delta R/R$)	n
1 μ M	40	55	12/105
10 μ M	20	50	74/107
30 μ M	15	76	114/134
100 μ M	10	101	68/93

Figure 3.23 - The time courses of the $[Ca^{2+}]_i$ responses to ATP in cultured sensory neurones.

The mean time course of the responses to A) 1 μ M ATP, B) 10 μ M ATP and C) 100 μ M ATP. The black bar indicates when ATP was present.



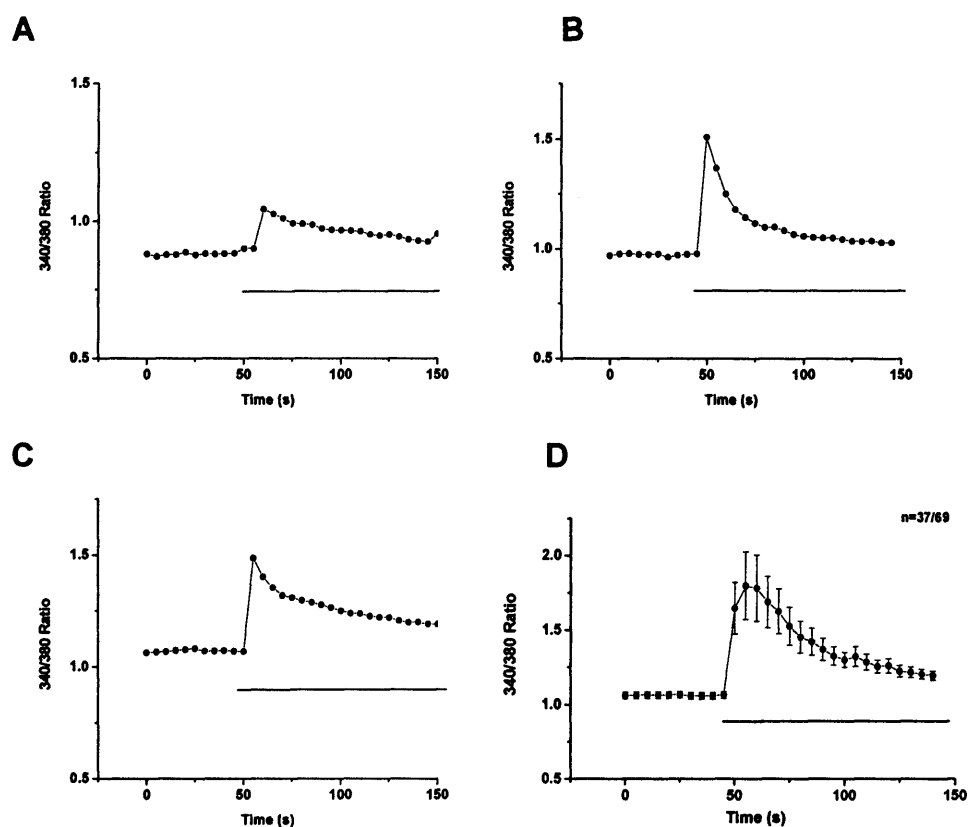


Figure 3.24 – Typical examples of calcium responses evoked in DRG neurones by 3 μ M ATP.

Panels A, B and C show typical examples of calcium responses evoked in sensory neurones by 3 μ M ATP. Panel D shows the mean calcium response profile for all the responders together with the s.e.m. The black line indicates the presence of ATP.

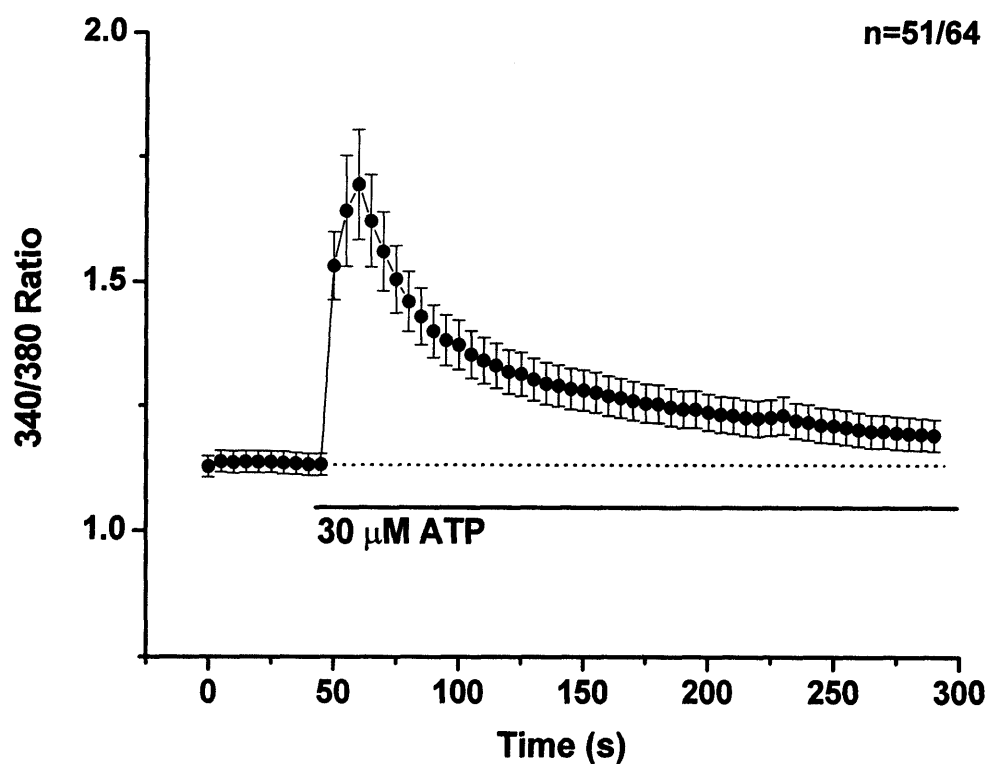


Figure 3.25 – The extended time course of the response to ATP.

The trendline on the extended time course illustrates that the response to 30 μ M ATP took more than 200 s to return to baseline. The black line represents the period during which the agonist was present. The time constant for the decay of the Ca^{2+} response was 30 s.

The source of calcium giving rise to the response of sensory neurones to ATP

Three quarters of the sensory neurones responded to the ATP challenge with a rise in intracellular calcium. To determine if intracellular calcium stores were involved in mediating the response to ATP, cells were challenged with 30 μM ATP in a calcium-free solution. A slight decrease in the percentage of neurones responding to 30 μM ATP (73% $n=70/96$) was observed in calcium free solution compared to the control (85% $n=114/134$) (see Figure 3.26). This decrease was not statistically significant ($p=0.27$). This suggested that the majority of cells responding to ATP utilised calcium from internal stores. Nevertheless the amplitude of the response in the calcium free solution was significantly ($p<0.001$) decreased and returned to resting level within the duration of the experiment (see Table 3.6 and Figure 3.27A).

Table 3.6 – The effect of the removal of external calcium on the characteristics of the response to ATP.

ATP concentrations	Time to Peak (s)	Amplitude % ($\Delta R/R$)	n
30 μM	15	76 \pm 9	114/134
30 μM (Ca^{2+} free)	15	40* \pm 8	70/96

The decrease in amplitude of the response to ATP upon removal of external calcium was found to be significant by means of Fisher's exact test * $p<0.001$.

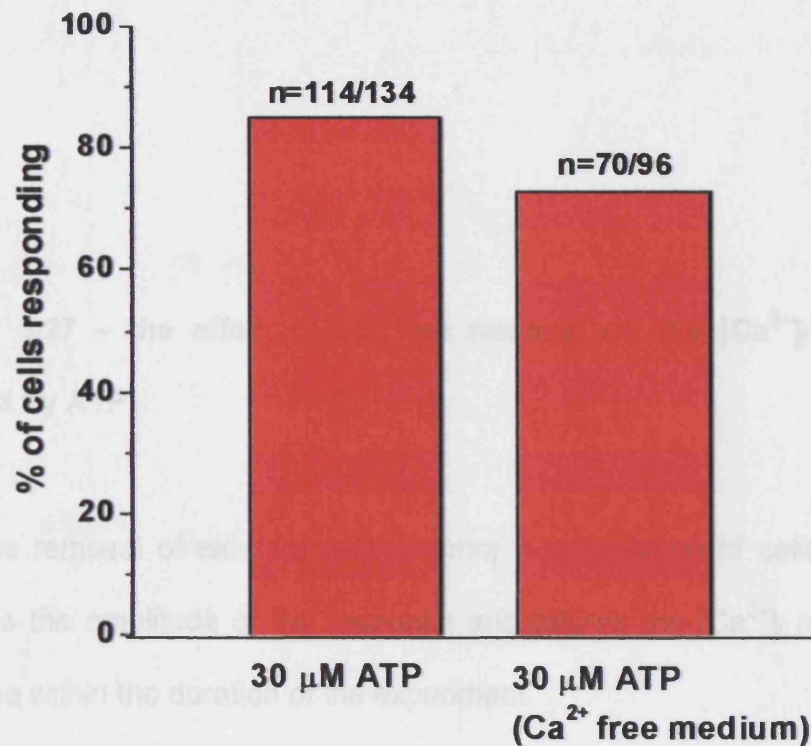


Figure 3.26 - Removal of calcium does not significantly affect the percentage of cells responding to ATP

There was a non-significant decrease ($p=0.27$) in the percentage of cells responding in the absence of calcium.

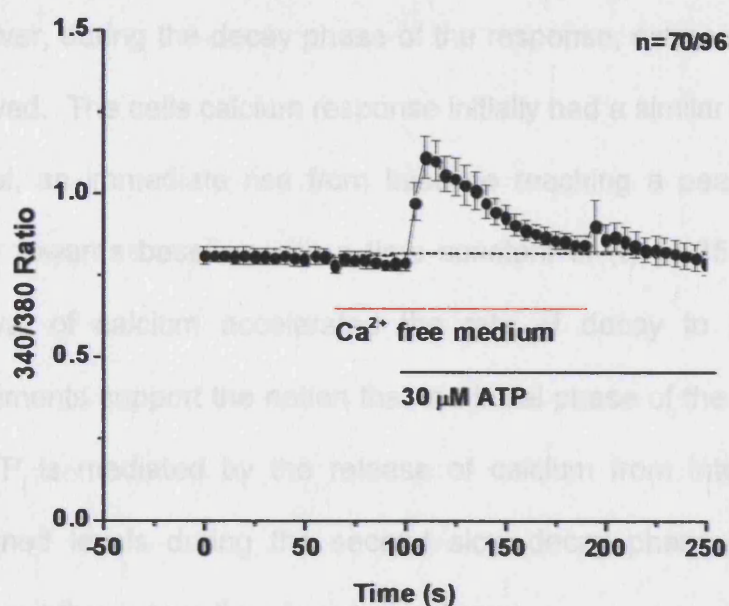
Figure 3.27 – the effect of calcium removal on the $[Ca^{2+}]_i$ response evoked by ATP

A) The removal of external calcium prior to stimulation of cells with ATP reduces the amplitude of the response and returns the $[Ca^{2+}]_i$ response to baseline within the duration of the experiment.

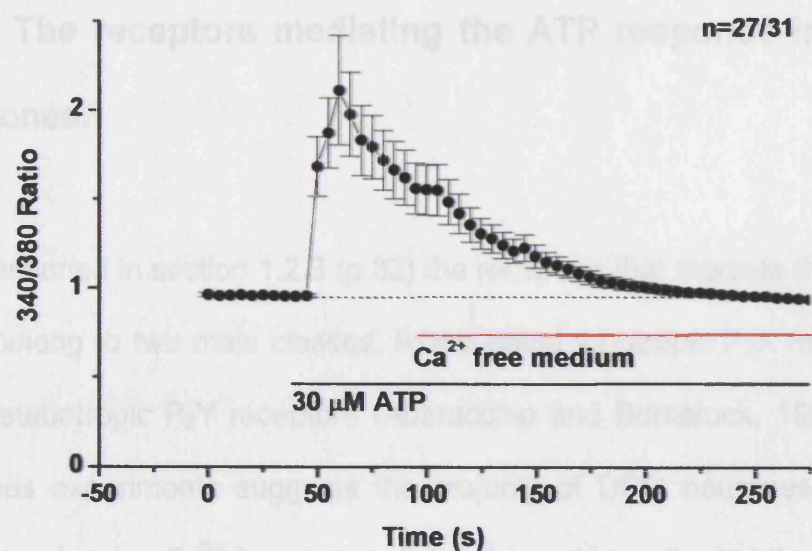
B) The removal of external calcium during the ATP response prevents the steady state and returns the calcium to basal level.

In both A and B the red line signifies the removal of calcium and the black line indicates the presence of ATP. The trendline gives an indication of the resting level of calcium. The effect seen upon changing the solutions (e.g. at 200 s in the Figure 3.27A) is a switching artefact.

A)



B)



In Figure 3.27 B cells were stimulated with ATP in the presence of calcium. However, during the decay phase of the response, extracellular calcium was removed. The cells calcium response initially had a similar time course to the control, an immediate rise from baseline reaching a peak within 15s then decay towards baseline with a time constant of (t_1 14.35s). However, the removal of calcium accelerated the rate of decay to baseline. These experiments support the notion that the initial phase of the calcium response to ATP is mediated by the release of calcium from internal stores while sustained levels during the second slow decay phase is due in part to calcium influx across the plasma membrane.

3.9 - The receptors mediating the ATP response in sensory neurones.

As mentioned in section 1.2.3 (p.32) the receptors that mediate the actions of ATP belong to two main classes, ligand-gated ionotropic P_2X receptors and the metabotropic P_2Y receptors (Abbracchio and Burnstock, 1994). As the previous experiments suggests the majority of DRG neurones respond to ATP by releasing Ca^{2+} from intracellular stores, I investigated the response of DRG neurones to selective P_2Y agonists. The agonists chosen were 2-methylthioadenosine triphosphate (2MeS-ATP) and 2-methylthioadenosine diphosphate (2MeS-ADP). The application of 30 μ M 2MeS-ATP to sensory neurones evoked a rise in $[Ca^{2+}]_i$ in 72% ($n=91/126$) of the sensory neurones which was not significantly different to the 85% ($n=114/134$ $p=0.151$) of cells

responding to 30 μM ATP (see Figure 3.28). This supports the notion that most sensory neurones responding to ATP possess P_2Y receptors.

However, as the specificity of the 2MeS-ATP agonist for the P_2Y receptors is controversial, it was decided to repeat the experiments with 2MeS-ADP which does not activate P_2X receptors (King and Townsend-Nicolson 2003). Application of 2MeS-ADP to sensory neurones also evoked a rise in $[\text{Ca}^{2+}]_i$ in a significant proportion of DRG neurones. As the concentration of 2MeS-ADP increased from 1 nM to 10 μM the percentage of cells responding rose from 6% (n=3/51) to 67% (n=58/87) (see Figure 3.29). There was no significant difference ($p=0.758$) between the fraction of cells responding to the highest concentration of ATP and that responding to the highest concentration of 2MeS-ADP.

These results are in agreement with the earlier conclusion (see p.130) that the calcium rise observed in sensory neurones in response to ATP is predominately mediated by P_2Y receptors.

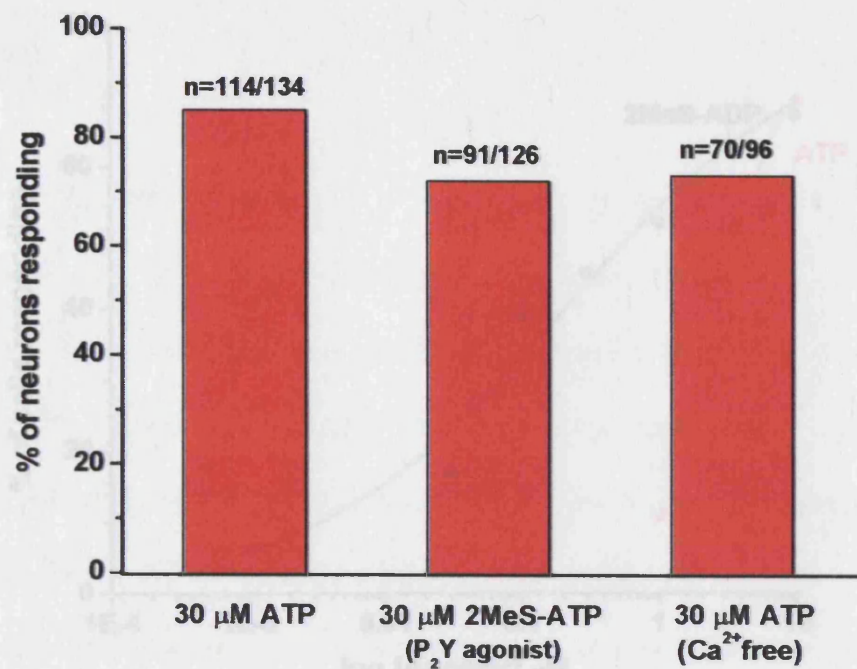


Figure 3.28 – The majority of the cells responding to ATP possess P_2Y receptors.

The figure illustrates the proportion of sensory neurones responding to 30 μ M ATP, 30 μ M 2MeS-ATP and 30 μ M ATP in 0 mM Ca^{2+} .

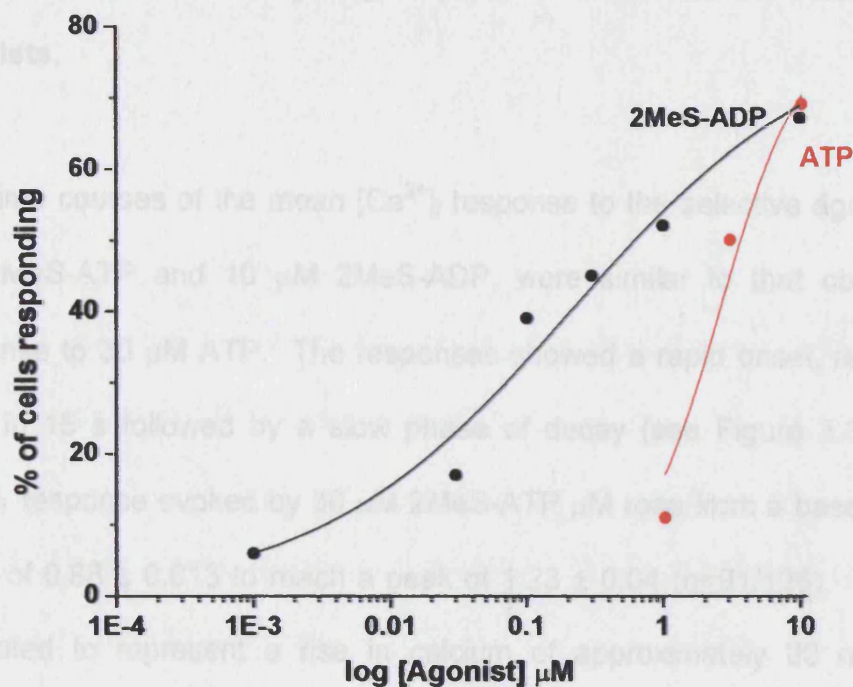


Figure 3.29 - The log dose response curves for 2-MeS-ADP and ATP

The graph shows the percentage of sensory neurones responding to increasing concentrations of 2 MeS-ADP within the range of 1 nM to 10 μM and ATP from 1 μM to 10 μM . The curves are fitted using the Hill equation to give slopes of 0.47 for the 2MeS-ADP response and 1.42 for the ATP response.

The time courses of the $[Ca^{2+}]_i$ responses to ATP and the selective P_2Y agonists.

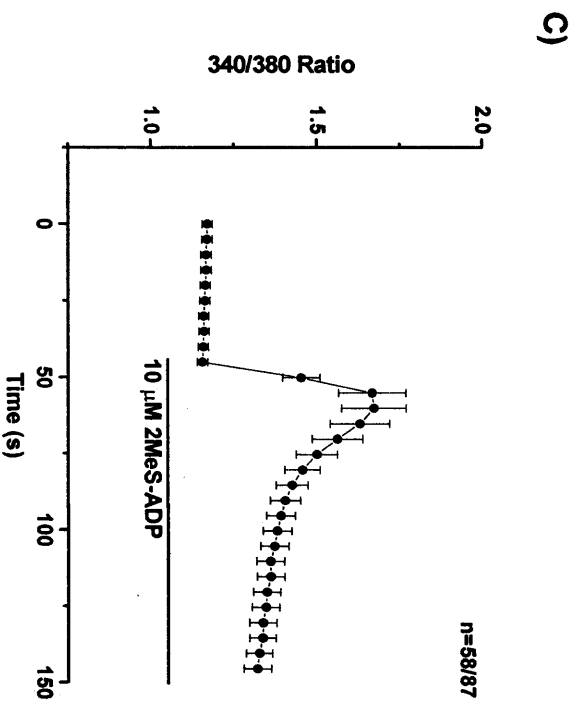
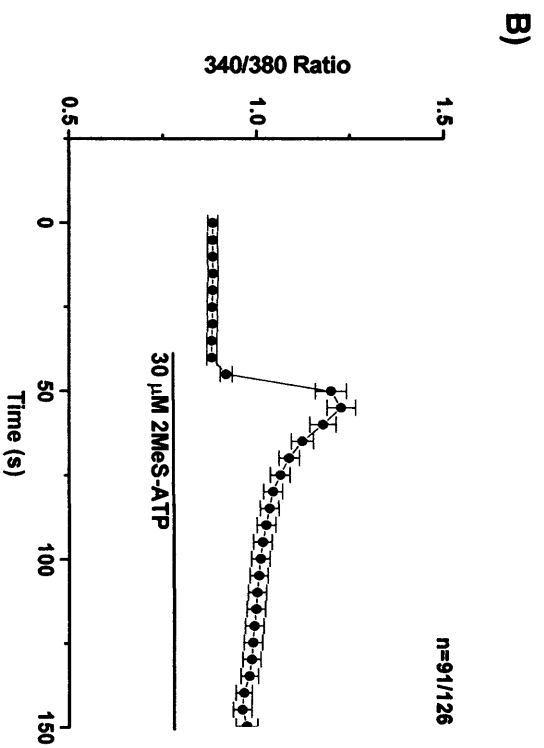
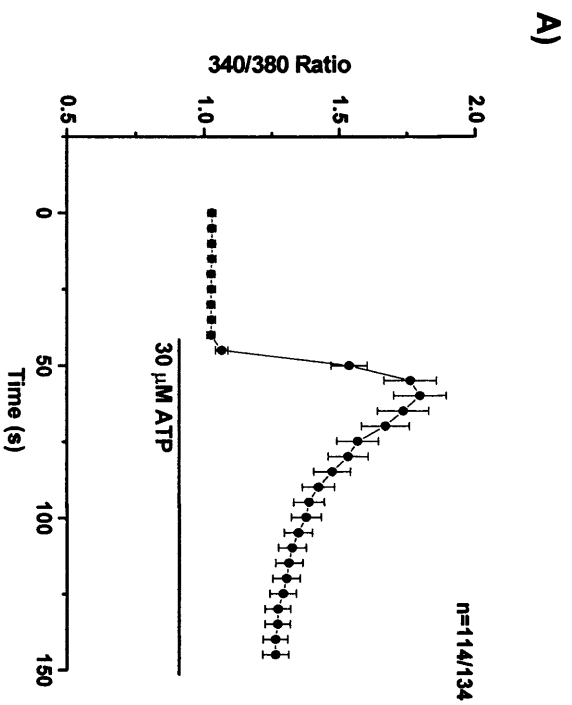
The time courses of the mean $[Ca^{2+}]_i$ response to the selective agonists, 30 μM 2MeS-ATP and 10 μM 2MeS-ADP, were similar to that obtained in response to 30 μM ATP. The responses showed a rapid onset, reaching a peak in 15 s followed by a slow phase of decay (see Figure 3.30). The $[Ca^{2+}]_i$ response evoked by 30 μM 2MeS-ATP rose from a baseline ratio value of 0.88 ± 0.013 to reach a peak of 1.23 ± 0.04 ($n=91/126$). This was estimated to represent a rise in calcium of approximately 80 nM. The response then decayed with a time constant of 19 s. The $[Ca^{2+}]_i$ responses to 10 μM 2MeS-ADP had an immediate onset rising from a baseline value of 1.17 ± 0.02 to a peak of 1.67 ± 0.1 ($n=58/87$). This ratio change represents a rise in $[Ca^{2+}]_i$ of approximately 90 nM. The response then decayed towards baseline with a time constant of 19 s. However, the amplitude of the $[Ca^{2+}]_i$ responses to 30 μM 2MeS-ATP and 10 μM 2MeS-ADP were considerably smaller than those in response to 30 μM ATP (see Table 3.7).

It is well known that some P_2Y receptors are coupled to PLC which activates calcium mobilisation from internal stores and PKC (see Figure 4.2). If P_2Y receptors mediate the majority of responses to ATP, application of the PLC inhibitor, U73122, should reduce the calcium response. Pre-treatment of cells with U73122 caused a significant reduction ($p=0.021$) in the amplitude of the calcium responses evoked (see Figure 3.31).

Figure 3.30 - The time course of the $[Ca^{2+}]_i$ responses to ATP and the P_2Y agonists 2MeS-ATP and 2MeS-ADP.

The DRG neurones were challenged with A) 30 μ M ATP B) 30 μ M 2MeS-ATP and C) 10 μ M 2MeS-ADP.

The black bar shows the addition of the agonist at 45 s and remains present for the duration of the experiment.



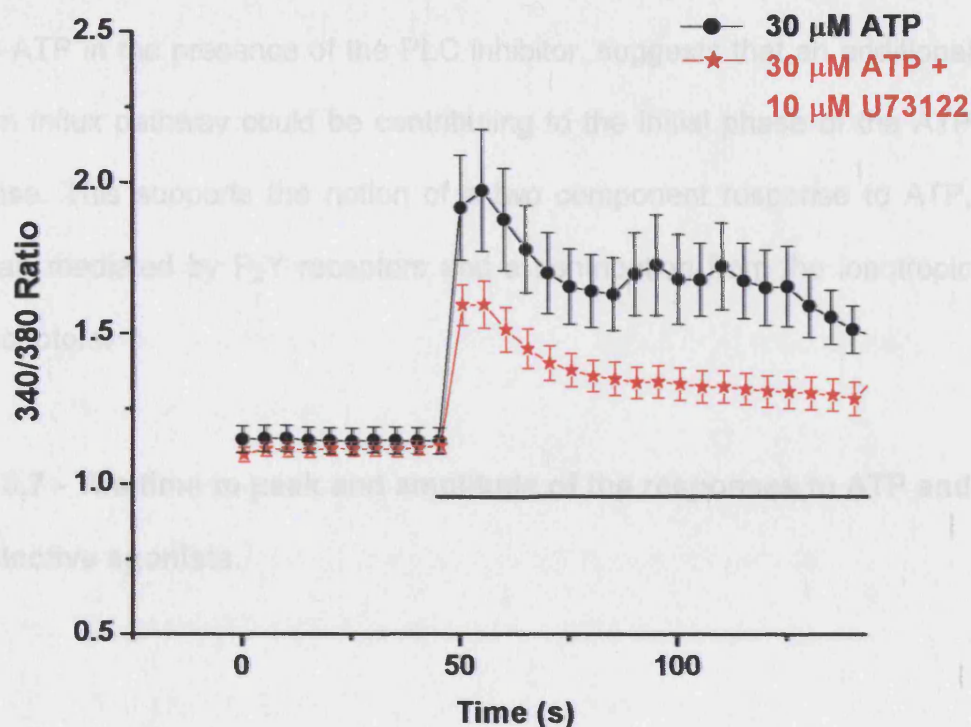


Figure 3.31 – The effect of the PLC inhibitor, U73122, on the $[\text{Ca}^{2+}]_i$ response to 30 μM ATP.

The time course of the responses was unchanged but the amplitude was reduced following pre-treatment with U73122.

The black line indicates the application of ATP.

The observed reduction in the amplitude of responses to the P₂Y agonists and to ATP in the presence of the PLC inhibitor, suggests that an additional calcium influx pathway could be contributing to the initial phase of the ATP response. This supports the notion of a two component response to ATP, with part mediated by P₂Y receptors and a contribution from the ionotropic P₂X receptors.

Table 3.7 - The time to peak and amplitude of the responses to ATP and the selective agonists.

Agonist concentration	Time to Peak (s)	Amplitude %($\Delta R/R$)	n =
30 μ M ATP	15	76 \pm 9	114/134
30 μ M 2MeSATP	15	38* \pm 4	91/126
10 μ M 2MeSADP	15	44 \pm 8	58/87
30 μ M ATP + 10 μ M U73122	10	30 \pm 7	29/51

The amplitudes of the calcium responses evoked upon stimulation with the selective receptor subtype agonists and with U73122 pre-treatment were compared with the control using the unpaired Welch corrected t test. The asterisk indicates a significant ($p < 0.001$) decrease in the amplitude of the response with respect to the control.

Summary of findings

- The potent algogen, ATP, evoked a rise in $[Ca^{2+}]_i$ in a large fraction of DRG neurones.
- The time course of the $[Ca^{2+}]_i$ response was concentration dependent i.e. as the concentration increased the amplitude increased and the time to peak decreased.
- The non significant decrease in the percentage of cells responding to ATP in a calcium free solution suggests the majority of cells responding to ATP utilised calcium from internal stores.
- The reduced amplitude of the $[Ca^{2+}]_i$ response when cells were pre-treated with the PLC inhibitor, U73122 and when challenged in calcium free solution suggests that the amplitude is mediated by calcium release from internal stores and an influx pathway.
- The large proportion of cells responding to the selective P_2Y agonists supported the involvement of these receptors in mediating the response to ATP.

4 – Discussion

Cultured DRG neurones were used as a model for the study of the chemosensitivity of primary afferent nerve endings. Sensory neurones were taken from all levels of the spinal cord. Thus, the cultured DRG cells contain cell bodies that would give rise to a diverse afferent population that subserve a variety of functions including proprioception, thermal sensation, mechanosensation and nociception. The subpopulations of DRG neurones can be categorised by their morphology, cell size, chemosensitivity and histological properties. Early studies using cytochemistry and electrophysiology identified two distinct subpopulations of neurones. The large diameter cells have soma which appear bright under phase microscopy. They give rise to thick myelinated axons. The smaller diameter cells ($< 30\ \mu\text{m}$) appear phase dark and give rise to unmyelinated or thinly myelinated processes.

Measurement of the diameters of the sample of neurones used for the calcium imaging experiments reported here showed that their diameters ranged from 9- $31\ \mu\text{m}$. The diameters of sensory neurones have previously been correlated with the fibre types arising from the cell (Harper and Lawson, 1985a). Cells of this size are known to give rise to thinly myelinated $A\delta$ fibres and unmyelinated C fibres (Harper and Lawson, 1985a).

This finding is consistent with the staining of DRG neurones with the neuronal markers IB₄, N52 and CGRP. The lectin IB₄ and CGRP antibody are known to

label small diameter unmyelinated sensory neurones. N52 antibody binds to cells rich in neurofilament thus are likely to be myelinated. The majority of cells in the field stained with either IB₄ or CGRP although a small number also stained for N52. The N52 positive cells are likely to give rise to myelinated A fibres. However, the neurones giving rise to the A α and A β fibres are known to have large diameter cell bodies which correspond to the large light cell population described by Harper and Lawson (1985b). The diameters of cells in this study are therefore likely to correspond to those giving rise to thinly myelinated A δ fibres or unmyelinated C fibres. The small diameter DRG neurones transmit nociceptive and thermal information to the spinal cord (Harper and Lawson, 1985a) whereas the large diameter cells transmit proprioceptive and tactile information (Nakamura and Strittmatter, 1996).

4.1 - Measurement of intracellular calcium

Calcium imaging experiments were performed on DRG neurones loaded with the fluorescent calcium dye, Fura-2. This dye was used to study the intracellular calcium responses evoked by sensory neurones upon the application of various inflammatory mediators. Dual wavelength dyes such as Fura 2 have two peaks either in their excitation or in their emission spectrum; upon substrate binding there is a shift in the intensity of both of the peaks. When Fura 2 binds calcium, the fluorescence emitted during excitation at 380 nM falls while the fluorescence emitted during excitation at 340 nM increases. The peak wavelength of the emission spectrum is not altered and its intensity is measured at 510 nM

(Grynkiewicz *et al.*, 1985). As Fura 2 is a ratiometric dye, a measure of free $[Ca^{2+}]_i$ can be obtained from the ratio of the fluorescence emitted at 340 nM divided by the fluorescence emitted at 380 nM. The use of Fura 2 prevents changes in intracellular dye concentration, bleaching, dye loss and cell thickness affecting measurements as these factors affect the intensity of signal proportionally at both wavelengths. Only the calcium concentration affects the intensities differentially. Thus the ratio of the excitation at 340 nM and 380 nM directly reflects free $[Ca^{2+}]_i$.

The fluorescence ratio values were calibrated (see Section 2.2) to allow the ratio changes to be correlated with the change in $[Ca^{2+}]_i$ (Grynkiewicz *et al.*, 1985)

$$[Ca^{2+}]_i = K_d [(R - R_{min}) / (R_{max} - R)] (S_{f2} / S_{b2})$$

This equation requires knowledge of the K_d value of the dye and the determination of the maximum ratio value and its minimum. The K_d value is an indicator of the dye's affinity for the agonist, in this case calcium. It depends on the microenvironment of the dye and the K_d value determined in a cuvette is only an approximate estimate of the actual value. Solutions containing excess calcium can be used to saturate the dye in the cells to give the R_{max} value. However, a true R_{min} value is very difficult to achieve as this requires a truly zero calcium solution. Measurements with calcium electrodes give values for such solutions in the range 10-30 nM. Moreover, calcium homeostasis is actively regulated by mitochondria, which are known to unload calcium at low cellular

calcium levels. Thus, to achieve a true R_{\min} value it is necessary to uncouple the mitochondria which will lead to a fall in intracellular pH, altering the K_d value. Consequently the R_{\min} value measured was probably too high. As this value affects the calculated free $[Ca^{2+}]_i$, the values given must be considered approximate.

4.2 - Chemosensitivity of the DRG neurones.

The cultured DRG neurones studied showed chemosensitivity to all three inflammatory mediators tested. Histamine, a well known pruritic, elicited a rise in $[Ca^{2+}]_i$ in just over a third of sensory neurones. The DRG neurones from Wistar rats were slightly less sensitive to histamine than neurones from Sprague Dawley rats. The potent algogen ATP evoked a rise in $[Ca^{2+}]_i$ in over three quarters of sensory neurones. My finding was within the range (40%-90%) of responding cells previously reported for the application of ATP to primary cultured sensory neurones (Bean, 1992, Grubb and Evans, 1999). The responses were either measured as a depolarising current by electrophysiology or indirectly by using calcium imaging to measure the change in $[Ca^{2+}]_i$. The last inflammatory mediator tested, 5-HT, evoked a rise in calcium in less than a fifth of sensory neurones.

This study also examined whether histamine responsive neurones could elicit calcium responses to algogenic agents. A sub-population of histamine responsive neurones also responded to ATP or 5-HT or capsaicin with a rise in

$[Ca^{2+}]_i$. However, a proportion of histamine responsive cells only responded to histamine. This suggests that there are sub-populations of histamine sensitive cells with varying sensitivity to other algogenic agents. This is supported by electrophysiological findings showing that histamine excites two subpopulations of C fibres: mechanoinensitive, thermally sensitive C fibres and mechanoinensitive, thermally insensitive C fibres (Schmelz *et al.*, 2003). However, these fibres can also be excited by capsaicin. A previous study using isolated DRG neurones showed that around 60% of small diameter neurones are sensitive to capsaicin. Moreover, the percentage of capsaicin sensitive cells that respond to histamine was significantly lower than the percentage of capsaicin insensitive cells responding (Nicolson *et al.*, 2002).

The time courses of the $[Ca^{2+}]_i$ responses to the three inflammatory mediators differed in their characteristics: time to peak and amplitude. The mean $[Ca^{2+}]_i$ response to histamine varied with the concentration applied. Application of low concentrations of histamine to the DRG neurones evoked a calcium response that reached a peak within 20 s then very slowly decayed towards baseline. Only at the highest concentration was there an increase in the amplitude of response and a decrease in the time to peak. The maximum ratio change was 0.71, which corresponds to an increase of 130 nM in $[Ca^{2+}]_i$.

The $[Ca^{2+}]_i$ responses to all ATP concentrations tested had an immediate onset, $[Ca^{2+}]_i$ rose to reach a peak within 10 seconds (at highest concentration). The $[Ca^{2+}]_i$ then slowly decayed towards baseline but, even after 200 seconds, $[Ca^{2+}]_i$

did not return to basal levels. Time to peak and amplitude of responses were concentration dependent. The maximum change in ratio was considerably higher than with histamine with a value of 1.09 which is approximately equivalent to a 200 nM increase in $[Ca^{2+}]_i$.

The time course of the $[Ca^{2+}]_i$ response to 5-HT differed from the responses observed with ATP and histamine application as it was not dependent on the concentration applied. Characteristically, it had a slow onset and the time to peak was approximately 40 seconds. This was considerably slower than the responses to ATP or histamine. Unlike the responses to histamine and ATP, the response to 5-HT did not decay. The $[Ca^{2+}]_i$ remained high for as long as the agonist was present. Therefore the response to 5-HT was an all or nothing event. The maximum change in ratio was 0.37, which corresponds approximately to a 70 nM rise in free $[Ca^{2+}]_i$.

The sensitivity of cultured DRG neurones to capsaicin has been shown to progressively decline over time (Nicolson 2000). This decline was shown by both a decrease in amplitude of the calcium response and decrease in the percentage of cells responding. After two to three days in culture the growth of neurites from the cell body is apparent (Lindsay *et al.*, 1991). There is evidence of receptor migration down these processes, which could account for the decrease in sensitivity of the cells bodies. In this study, the cells were kept in culture for a maximum of three days. However, no significant decreases in the proportion of cells responding or alterations in the calcium response

characteristics were observed to any of the agonists tested: histamine, 5-HT and ATP.

The origin of the rise in calcium

As mentioned in the Introduction, the cell has two sources of calcium to initiate calcium signalling, an infinite extracellular source and a finite intracellular source. The intracellular sources include the mitochondria and the endoplasmic reticulum where calcium release is mediated by two channels: the IP₃ receptor gated channel and the ryanodine receptor gated channel. Both channels have been reported to be present in the DRG neurones (Kostyuk and Verkhratsky, 1994). A previous study in rat DRG neurones, cultured under similar conditions to those used here, showed that the application of ryanodine failed to elicit [Ca²⁺]_i responses in any neurones tested (Nicolson, 2000). The rise in [Ca²⁺]_i was therefore most likely to be mediated via PLC/IP₃ second messenger pathway. This was confirmed by experiments where the rise in calcium was blocked by the pre-treatment of cells with the PLC inhibitor, U73122.

The mechanisms mediating intracellular calcium responses to histamine varied with concentration. The calcium signal obtained in response to low concentrations of histamine was initially due to calcium release from IP₃ sensitive stores then sustained probably by activation of a store depletion entry pathway (Nicolson *et al.*, 2002). However, in the presence of high histamine concentrations the evoked calcium response was mediated by store release and a calcium entry pathway that was independent of calcium mobilisation from the

IP₃ sensitive stores. The processes involved in mediating the calcium responses of DRG neurones to ATP and 5-HT had not previously been determined.

The removal of extracellular calcium prevents the influx of calcium, therefore any [Ca²⁺]_i response in the calcium free media must be due to calcium release from internal stores. The percentage of cells responding to either ATP or 5-HT with a rise in [Ca²⁺]_i were not significantly decreased in the absence of calcium. This suggests that, like histamine, the [Ca²⁺]_i responses evoked by these agonists were mediated by a process requiring the release of calcium from intracellular stores. The receptors that mediate these responses are discussed below.

The removal of external calcium did, however, affect the time courses and amplitudes of the calcium responses evoked by both mediators. The [Ca²⁺]_i responses to ATP in the absence of extracellular calcium had a similar rapid onset reaching a peak in 15 seconds. However, there was a significant reduction in amplitude from 76±9% to 40±8% (p=0.001) of the response. Moreover, the response showed a faster rate of decay and returned to the baseline. This is consistent with the view that the initial phase of the response was mediated by release of calcium from internal stores. Nevertheless, the reduction in amplitude seen in calcium free solution suggests that there was a significant contribution from a calcium influx pathway (e.g. involvement of P₂X receptors or voltage gated calcium channels). The slow decaying component of the response was calcium dependent and thus could be mediated by store-operated channels

which are activated following depletion of intracellular calcium stores (Berridge, 1995, Putney *et al.*, 2001). Calcium release activated calcium channels have low conductance and remain open over a longer time scale than voltage gated Ca^{2+} channels. Therefore activation of these channels could account for the prolonged decay.

Consistent with my findings, a recent study showed that application of extracellular ATP evoked an increase in $[\text{Ca}^{2+}]_i$ in a subpopulation of small diameter rat DRG neurones. These responses were mediated by the release of calcium from internal stores and an influx of calcium (Sanada *et al.*, 2002). The endoplasmic reticulum calcium stores can be depleted by thapsigargin, an agent that specifically and irreversibly inhibits calcium uptake through endoplasmic reticulum calcium pumps (Thastrup *et al.*, 1990, Premack *et al.*, 1994). Pre-treatment of DRG neurones with thapsigargin blocked the increase $[\text{Ca}^{2+}]_i$ in response to ATP and that to UTP (Sanada *et al.*, 2002). The specific PLC inhibitor, U73122, prevents the formation of IP_3 and thus the release of calcium from internal stores. When cells were pre-treated with U73122 there was a significant reduction in the amplitude of the calcium response evoked by cells upon stimulation with ATP (see Results p.141). This is consistent with the notion that the response of sensory neurones to ATP is primarily mediated by release of calcium from internal calcium stores implicating the involvement of the G protein coupled P_2Y receptors (Abbracchio and Burnstock, 1994). However the failure of U73122 to totally abolish the calcium response to ATP suggests that a calcium influx pathway is also involved.

The time course of $[Ca^{2+}]_i$ responses to 5-HT was also affected by the removal of external calcium. Unlike the responses to ATP, when cells were challenged with 5-HT in the absence of external calcium there was a significant *increase* in amplitude of the $[Ca^{2+}]_i$ responses from $41 \pm 8\%$ to $95 \pm 28\%$ over basal levels ($p=0.01$). This result was surprising and the underlying mechanisms remain unclear. When cells were pre-treated with PLC inhibitor, U73122, then challenged with 5-HT there was a significant reduction in the amplitude of the calcium response. The initial phase of the response to 5-HT was mediated by release of calcium from internal calcium stores. The initial decay after the peak in the response to 5-HT in a calcium free solution suggests that the sustained component seen in responses in the presence of calcium is due to the activation of a calcium influx pathway. As for the slow component of the response to ATP, the sustained component of the response to 5-HT could be mediated by the activation of capacitative calcium entry.

4.3 - The receptors mediating the responses to histamine, 5-HT and ATP.

In DRG neurones, the $[Ca^{2+}]_i$ responses evoked by the application of histamine were found to be mediated by the H_1 receptor (Nicolson *et al.*, 2002). The selective H_1 receptor antagonist, mepyramine, abolished $[Ca^{2+}]_i$ response to low concentrations of histamine and significantly reduced the response to high concentrations (10^{-2} M). In contrast, selective antagonists of the H_2 (cimetidine)

and H₃ (thioperamide) receptor subtypes had no significant effect on the response to histamine at any concentration tested (Nicolson, 2000).

Known immunohistochemical markers were used to characterise the neurones that expressed the H₁ receptor. As noted earlier, the cultured DRG neurones contained a number of subpopulations of histamine responsive neurones, which varied in their sensitivity to other algogenic agents. Subpopulations of H₁ receptor expressing neurones showed co-localisation with the nociceptive fibre markers CGRP and IB₄. Thus, suggesting that H₁ receptor expressing neurones are likely to give rise to unmyelinated, nociceptive fibres. However, there was a population of neurones that only stained for the H₁ receptor and another small proportion of H₁ positive neurones that showed co-localisation with the neurofilament marker, N52. This is consistent with the notion that some histamine sensitive neurones give rise to myelinated fibres.

The calcium response to 5-HT is mediated by 5-HT₂ receptors.

Seven families of 5-HT receptors have been identified (Hoyer *et al.*, 1994). All except one (5-HT₃) of the 5-HT receptors couple to guanine nucleotide binding proteins (G proteins) and regulate the intracellular concentration of second messengers that regulate cellular functions (see Figure 4.1). Studies using immunohistochemistry, *in situ* hybridisation and reverse transcription-PCR demonstrate that DRG neurones express multiple 5-HT receptor subtypes. mRNA for 5-HT_{1F}, 5-HT_{2C}, 5-HT₄, 5-HT_{5A}, 5-HT_{5B} have been found in DRG neurones. Radioligand binding studies showed that functional receptors existed

for 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT₃ on DRG neurones (Chen *et al.*, 1998, Pierce *et al.*, 1996, Nicholson *et al.*, 2003).

In the present study, the stimulation of DRG neurones with 5-HT in the absence of external calcium showed that the responses of most cells involved the release of calcium from internal stores. Of the known classes of 5-HT receptors only the 5-HT₂ receptor subtypes are positively coupled to G_{q/11} which activates PLC/IP₃ pathway (see Figure 4.1). This leads to the mobilisation of calcium from internal stores and thus an elevation of cytosolic calcium concentration. 5-HT₂ and 5-HT₃ receptor selective agonists were applied to sensory neurones to determine their degree of involvement in mediating the intracellular calcium responses evoked by DRG neurones. The percentage of cells responding to the 5-HT₂ agonist, α Me5-HT, was not significantly different to the control. However, only a small fraction of cells responded to the application of the 5-HT₃ selective agonist, MCPB. This fraction was similar to the percentage of cells responding to 5-HT in the presence of the specific PLC inhibitor, U73122.

There are reports indicating that peripheral 5-HT induced nociceptive responses are largely mediated by 5-HT₃ receptors located on sensory nerve terminals (Orwin and Fozard, 1986, Giordano and Dyche, 1989). Electrophysiological studies have shown that activation of 5-HT₃ receptors causes a depolarisation that is due to a transient inward current resulting from the opening of a non selective cation channel (Robertson and Bevan, 1991). Functional 5-HT₃

receptors are expressed by DRG neurones. Nevertheless, our findings show that only a small fraction of DRG neurones increase $[Ca^{2+}]_i$ in response to 5-HT by activation of ionotropic 5-HT₃ receptors. Our data strongly supports the notion that in sensory neurones the calcium response to 5-HT is predominately mediated by the 5-HT₂ receptors

The time course of the $[Ca^{2+}]_i$ response to the 5-HT₃ receptor agonist, MCPB, had an amplitude comparable to that seen in cells stimulated with 5-HT. Interestingly, the initial peak of the response to α Me5-HT was significantly greater than that in response to 5-HT yet it was comparable to the peak amplitude of the calcium response to 5-HT in the absence of calcium. The difference between the responses resides in the initial kinetics of the calcium response as all three responses (5-HT, α Me5-HT, Ca^{2+} free 5-HT) reach a similar end point. The difference in these responses may reflect the fact that many different subtypes of 5-HT receptors are expressed by DRG neurones (see above). Except for 5-HT₃ receptors, all are G protein coupled. 5-HT₂ receptors are coupled to G_q/ G₁₁ and activate PLC. All others regulate adenylyl cyclase by either up regulating or down regulating its activity. In the experiments discussed here 5-HT was used at a maximal concentration and will activate many different 5-HT receptor subtypes in addition to 5-HT₂. The response to 5-HT could reflect the modulatory effect of another 5-HT receptor subtype on IP₃ production which may alter the kinetics of the release of calcium

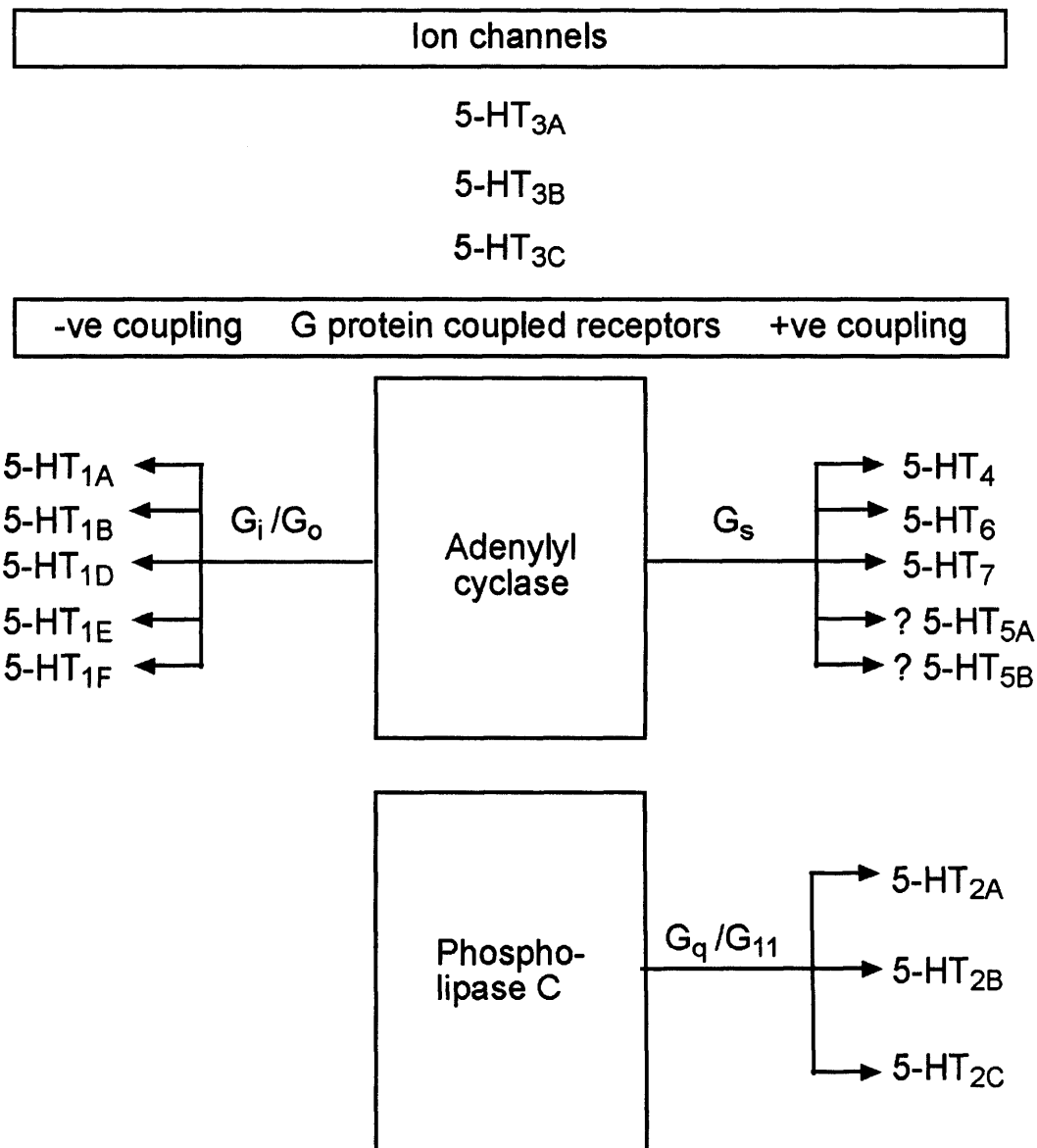


Figure 4.1- The classes of 5-HT receptor and their coupling

(Adapted from Hoyer *et al.*, 2002)

from internal stores. However, the precise explanation for the difference in kinetics remains unresolved.

Of the three known 5-HT₂ receptor subtypes, 5-HT_{2a} and 5-HT_{2c}, have been shown to be present on DRG neurones (Chen *et al.*, 1998, Pierce *et al.*, 1996). The accumulating evidence supports the involvement of 5-HT_{2A} receptor in the mediation of pain and hyperalgesia. In behavioural studies, intradermal injections of 5-HT_{2A} receptor agonist, α Me5-HT, into the rats ipsilateral hindpaw resulted in a significant reduction in the paw withdrawal latency to radiant heat stimuli. This was not seen following the application of 5-HT_{1A} (8-OH-DPAT) or 5-HT₃ (2Me5-HT) receptor selective agonists (Tokunaga *et al.*, 1998). Pre-treatment with the 5-HT_{2A} receptor antagonist, ketanserin, attenuated the hyperalgesia induced by injections of 5-HT in a dose dependent manner (Tokunaga *et al.*, 1998). The behavioural responses (e.g. lifting and licking) induced by 5-HT or 5-HT induced potentiation of pain, produced by other inflammatory mediators, are mediated by 5-HT_{2A} receptors (Abbott *et al.*, 1996).

In vivo, 5-HT₂ receptors are present on mast cells and platelets. However the 5-HT induced effects described above are mediated by a direct pathway as the elimination of all known indirect pathways that contribute to hyperalgesia failed to inhibit 5-HT induced hyperalgesia (Taiwo and Levine, 1992). In rat glabrous skin, around a third of unmyelinated peripheral afferent fibres were labelled with antibodies directed against the 5-HT_{2A} receptors (Carlton and Coggeshall,

1997). The mRNA for 5-HT_{2A} receptors is expressed on a small subset around 10% of small diameter CGRP synthesising DRG neurones (Okamoto *et al.*, 2002). This anatomical evidence supports the idea that 5-HT can have a direct effect on primary afferent nerve endings. Expression of Fos-like immunoreactivity has been used as a marker of neuronal activation (Doi-Saika *et al.*, 1997). Peripheral injection of 5-HT and 5-HT_{2A} agonist, α Me5-HT, induced Fos-like immunoreactivity in second order neurones in the dorsal horn that was not seen following administration of the 5-HT₃ agonist, m-CPG. The Fos-like immunoreactivity was reduced by 5-HT_{2A} receptors antagonist ketanserin. This evidence supports the notion that 5-HT₂ receptors play a role in the mediation of pain and hyperalgesia.

Receptors mediating the response to ATP

In man the application of ATP to a blister base elicits a sensation of pain (Bleehen and Keele, 1977). Subsequently, patch clamp studies on isolated rat sensory neurones showed that ATP gated an inward cationic current resulting in rapid depolarisation (Krishtal *et al.*, 1983, Bean, 1992, Grubb and Evans 1999). Thus, ATP released from damaged tissue has an important role in the initiation of noxious signals. Its actions are mediated by the activation of specialised cell surface receptors, collectively called P₂ purinoceptors, expressed by primary afferent fibres (Burnstock and Kennedy, 1985). The P₂ purinoceptors can be subdivided into two major classes, P₂X which are ligand gated cation channels

and P₂Y which are G-protein coupled receptors (Abbracchio and Burnstock, 1994).

The P₂X receptor family contains 7 homomeric (P₂X₁₋₇) and 5 heteromeric assemblies (P₂X_{1/2}, P₂X_{1/5}, P₂X_{2/3}, P₂X_{2/6}, P₂X_{4/6}) characterised by their biophysical and pharmacological properties (Burnstock, 1999). P₂X receptors are cation selective channels significantly permeable to calcium with almost equal permeability to sodium and potassium (Ralevic and Burnstock, 1998). mRNA for P₂X₁₋₆ receptors is found in dorsal root and trigeminal ganglia, while that of P₂X₇ receptors is found exclusively in cells of the immune system (MacKenzie *et al.*, 1999).

The broadly distributed P₂Y receptors are coupled to heterotrimeric G proteins which activate a variety of intracellular second messenger systems, such as PLC β and adenylyl cyclase. Of the 14 P₂Y receptor-like DNA sequences that have been cloned, only eight are accepted as functional mammalian P₂Y receptors (P₂Y₁, P₂Y₂, P₂Y₄, P₂Y₆, P₂Y₁₁, P₂Y₁₂, P₂Y₁₃, P₂Y₁₄,) (Boarder and Hourani, 1998). The P₂Y₁, P₂Y₂, P₂Y₄, P₂Y₆ and P₂Y₁₁ are strongly coupled to G_{q/11} which activates the PLC / IP₃ pathway, which ultimately leads to an increase in intracellular calcium (see Figure 4.2). Using reverse transcription PCR, mRNA for metabotropic P₂Y₁, P₂Y₂, P₂Y₄ and P₂Y₆ receptors have been shown to be expressed on adult rat DRG neurones (Sanada *et al.*, 2002). ADP, UTP

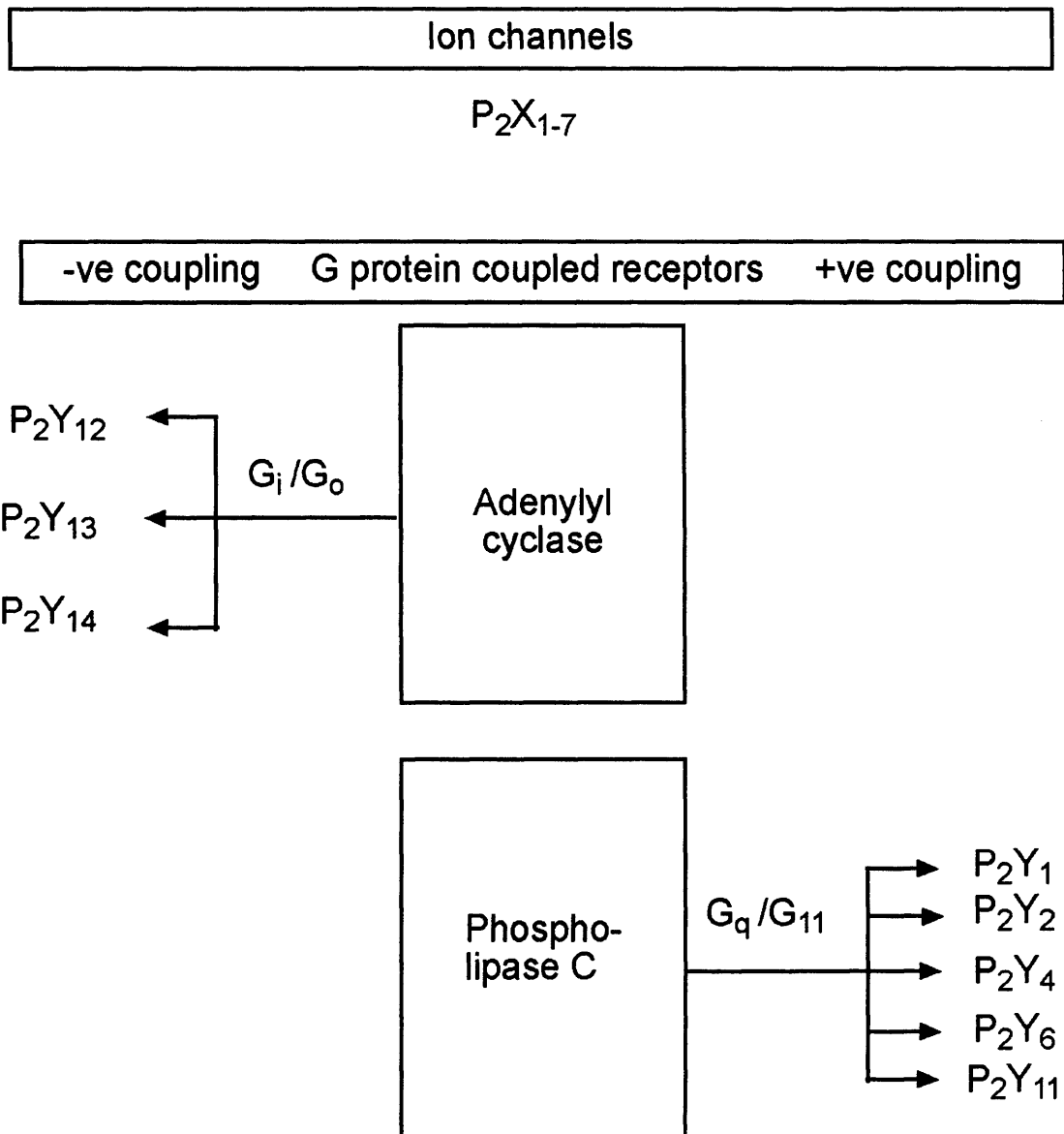


Figure 4.2 – The classes of purinergic receptors and their coupling.

and UDP are selective agonists for P₂Y receptors and thus are used to characterise the functional expression of these receptors. Moreover the potency of nucleotides triphosphates and diphosphates varies at different P₂Y receptor subtypes. P₂Y₁ receptors are adenine nucleotide preferring thus are fully activated by ATP and ADP. P₂Y₂ and P₂Y₄ receptor subtypes are triphosphate preferring and are equally responsive to ATP and UTP (Ralevic and Burnstock, 1998). P₂Y₆ receptors are responsive to UTP and UDP (Lustig *et al.*, 1993, Ralevic and Burnstock, 1998, Bogdanov *et al.*, 1998, Filippov *et al.*, 1999).

Early investigations into the role of ATP receptors in pain and inflammatory pathways primarily focused on the P₂X₃ receptor as this receptor is expressed at high levels exclusively by a subset of small diameter nociceptive sensory neurones (Chen *et al.*, 1995, Lewis *et al.*, 1995, Burnstock and Wood, 1996). This was supported by evidence that noxious actions of ATP upon application to a human blister base (Bleehen and Keele 1977) or when injected directly into human skin could be inhibited by P₂X receptor antagonists (Richardson *et al.*, 1985). However, recent reports using gene knockout methods (Cockayne *et al.*, 2000, Souslova *et al.*, 2000), antisense oligonucleotides technologies (Barclay *et al.*, 2002) and a novel selective P₂X₃ antagonists (Jarvis *et al.*, 2002) all suggest that P₂X₃ receptors are more likely to be involved in chronic conditions particularly chronic inflammation and neuropathic pain. However, it has come to light that ATP and related nucleotides ADP,UTP,UDP may influence sensory nerve activity by activating P₂Y receptors. Now attention has turned to P₂Y receptors and their possible

involvement in pain. The following data shows the involvement of P₂Y receptors in mediating the response elicited by DRG neurones upon stimulation with ATP. The findings reported here showed that the percentage of cells responding to ATP was unaffected by the removal of external calcium. This suggests that most cells responded to ATP via receptors that are coupled to the PLC-IP₃ pathway, implicating the involvement of metabotropic P₂Y receptors. To determine what fraction of sensory neurones mediated their response by this mechanism the cells were challenged with P₂Y selective agonists (2MeS-ATP, 2MeS-ADP). The percentage of cells responding to each of the selective P₂Y receptor agonists was not significantly different from that of the control ATP concentration. The P₂ Y selective agonist, 2MeS-ADP, produced responses over a greater range of concentrations resulting in a Hill coefficient of less than one (n_H 0.47) suggesting the involvement of more than one P₂Y receptor subtype. A similar study showed that application of equal concentrations of ATP and UTP to DRG neurones in the absence of extracellular calcium evoked responses in a similar percentage of cells (Sanada *et al.*, 2002). Note that the only P₂Y receptors that are equally sensitive to ATP and UTP are the P₂Y₂ and P₂Y₄ receptors. This suggests that the P₂Y₂ and P₂Y₄ receptors may mediate the rise in [Ca²⁺]_i observed with application of ATP to DRG neurones.

The time courses of the [Ca²⁺]_i responses to the two P₂Y agonists showed considerable similarity to the [Ca²⁺]_i response to ATP. All the responses reached their peak in the same time. However, there was a significant reduction in amplitude of the calcium responses to the two selective receptor agonists.

This reduction in $[Ca^{2+}]_i$ response amplitude was also seen when the cells were pre-treated with the PLC inhibitor U73122 and when challenged with ATP in a calcium free solution. This evidence supports the present findings of the involvement of metabotropic P_2Y receptors acting on the PLC/ IP_3 pathway to release internal stores of calcium. However, there was a small contribution to this response from a calcium influx pathway (e.g. P_2X receptors or voltage gated calcium channels).

There is some evidence to support a role for P_2Y_2 receptors in the mediation of nociception and in the sensitisation of DRG neurones to other inflammatory mediators. In rat sensory neurones application of low concentrations of ATP alone does not increase the release of substance P and CGRP. However, when ATP is applied prior to application of capsaicin there was a significant increase in the quantity of neuropeptides released by capsaicin (Huang *et al.*, 2003). This sensitising role of ATP is believed to be mediated by activation of PKC through P_2Y receptors, possibly by P_2Y_2 receptor subtype (Huang *et al.*, 2003).

Intense or sustained stimulation increases the responsiveness of both primary afferents and second order neurones in the spinal cord. Initially these effects are the result of modulation of existing receptors and ion channels however, in the long term they will also involve changes in gene expression (Woolf and Salter, 2000). Activation of transcription factors may regulate these changes and the calcium and cAMP response element binding protein (CREB) transcription factor may regulate the change in sensory neurone phenotype in

response to injury and inflammation (Ji and Woolf 2001). ATP and UTP induce CREB phosphorylation in a subset of adult DRG neurones, which have the characteristics of nociceptors, by the activation of P₂Y₂ receptors (Molliver *et al.*, 2002). However, the activation of P₂X receptors could not induce CREB phosphorylation. CREB phosphorylation is calcium dependent. UTP evoked CREB phosphorylation is only blocked by the removal of external calcium and by inhibition of internal calcium store release. This is consistent with my finding that the amplitude of the calcium response was mediated by two processes: calcium influx and calcium release from internal stores.

In situ hybridisation revealed that 77% of sensory neurones expressed P₂Y₂ mRNA (Molliver *et al.*, 2002). However, only 40% of these neurones responded to ATP or UTP by phosphorylating CREB. Interestingly, nearly all the neurones which showed CREB phosphorylation were peripherin positive and small in diameter. Immunostaining with markers used to identify nociceptive fibres showed that of the neurones that were positive for CREB phosphorylation 34% also stained positive for CGRP while 38% of these neurones bound IB₄. This suggests that P₂Y₂ receptors are expressed on a larger population than those expressing P₂X₃ receptors which are mainly IB₄ binding neurones (Bradbury *et al.*, 1998, Vulchanova *et al.*, 1998). P₂Y₂ receptors have also been shown to mediate the slow onset and sustained trains of action potentials observed in nociceptors activated by ATP (Molliver *et al.*, 2002).

In summary, my findings show that most sensory neurones respond to ATP via activation of P₂Y receptors. This is consistent with the current evidence that the P₂Y receptors have a role in the activation of sensory neurones and in the mediation of pain.

4.4 – Mechanisms of PGE₂ induced sensitisation of sensory neurones by inflammatory mediators.

Adaptation to a maintained stimulus is a general feature of sensory systems. However, there are exceptions to this rule: both itch and pain sensations do not adapt to stimuli that are potentially tissue damaging, but instead frequently exhibit sensitisation. This is observed in behavioural studies as a heightened perception of pain in man (Handwerker and Reeh, 1991) and as increased nocifensive behaviour (e.g. licking and lifting) to suprathreshold stimuli in animals (Ferreira *et al.*, 1978, Hamilton *et al.*, 1999). Upon tissue damage or inflammation a range of mediators including 5-HT and prostaglandins are released. Some of these mediators then interact with receptors or ion channels on sensory nerve endings to produce sensitisation. In this study the sensitising actions of PGE₂ on the response to histamine and 5-HT have been examined.

The ability of PGE₂ to alter pain perception by sensitising sensory neurones to noxious chemicals, thermal and mechanical stimuli is well known. Early work demonstrated that intradermal injection of PGE₁ or PGE₂ into human skin did not elicit pain despite producing a pronounced wheal and flare (Ferreira, 1972).

However, when PGE₁ was injected prior to bradykinin, the pain induced by bradykinin was greatly enhanced (Ferreira, 1972). Animal studies by themselves cannot elucidate the mechanism of action but showed that hyperalgesia could be induced by intradermal injections of activators of cAMP pathways such as forskolin and dibutylcAMP (Ferreira and Nakamura, 1979, Taiwo and Levine, 1991, Taiwo *et al.*, 1989). The forskolin-induced hyperalgesia could be prolonged by co-injection of phosphodiesterase inhibitors (which prevent breakdown of cAMP) (Taiwo *et al.*, 1989) and attenuated by inhibitors of PKA (e.g H89) (Taiwo and Levine, 1991). Thus, behavioural models of nociception suggested that the increased sensitivity to noxious stimuli induced by prostaglandins was mediated in part by the activation of cAMP transduction cascade.

PGE₂ pre-treatment of cultured sensory neurones showed a significant increase in intracellular levels of cAMP (Nicolson, 2000). This supported the notion that cAMP is involved in the PGE₂ induced sensitisation. However, the specific role that cAMP played in sensitisation was unknown.

An earlier study showed that PGE₂ pre-treatment increased the proportion of DRG neurones which responded to bradykinin (Smith *et al.*, 2000). The sensitisation reduced the response threshold shown by elicitation of [Ca²⁺]_i responses to low concentrations of bradykinin in initially unresponsive cells and an increased total number of responsive cells. This sensitisation could be

blocked by pre-treatment with a PKA inhibitor, H89. Thus, PKA plays an important role in the PGE₂ sensitisation of bradykinin evoked [Ca²⁺]_i responses.

Nicolson (2000) showed that PGE₂ was also capable of sensitising a sub-population of rat DRG neurones to histamine. The percentage of cells responding to low concentrations of histamine was increased with PGE₂ pre-treatment. The sensitisation of the histamine response could be mimicked by cAMP analogues e.g. (8-bromo cAMP) and inhibited by adenylyl cyclase blocker (e.g. tetrahydro-furyl-adenine) (Nicolson, 2000). This suggested the involvement of cAMP but did not fully elucidate the mechanism by which cAMP induced sensitisation. The present study showed that the pre-treatment of cells with the PKA inhibitor, H89, prior to PGE₂ pre-treatment blocked the sensitisation of the histamine response. This was consistent with the mechanism determined for PGE₂ induced sensitisation of sensory neurones to bradykinin.

The sensitised response

PGE₂ alone had no effect on [Ca²⁺]_i in DRG neurones which is consistent with previous reports that at concentrations which produce sensitisation PGE₂ does not directly activate sensory fibres. However, Smith *et al.*, (2000) have reported a direct effect of PGE₂ on [Ca²⁺]_i in sensory neurones but these experiments were performed on cultured DRG neurones isolated from neonates and at a temperature of 37°C. Most electrophysiological experiments performed *in vitro* are conducted at room temperature (20-24°C) perhaps explaining why there is

very limited evidence of the direct activation by prostaglandins in sensory neurones. The concentration of PGE₂ used in these experiments to produce sensitisation are consistent with previous studies reporting the sensitisation of the bradykinin response in culture sensory neurones and within the range present in inflammatory exudates (Nicol and Cui, 1994, Smith *et al.*, 2000).

Pre-treatment with PGE₂ increased the proportion of neurones that responded to histamine with a rise in [Ca²⁺]_i however, it did not affect the profile of the [Ca²⁺]_i response obtained to histamine. The sensitised response induced by cAMP analogues similarly had no effect on the time course or amplitude. Thus, the sensitising effects were due to a reduction in the threshold for a response rather than an enhancement of the calcium response. This is unlike the PGE₂ induced sensitisation of the bradykinin response obtained in cultured DRG neurones where both the number of cells responding was increased and the bradykinin evoked [Ca²⁺]_i response was enhanced (Stucky *et al.*, 1996, Smith *et al.*, 2000). PGE₂ had no effect on histamine's induced increase in IP₃ formation and thus it's ability to activate the PLC/IP₃ transduction pathway.

Mechanism mediating the sensitisation of the response to 5-HT

Co-injections of 5-HT and PGE₂ were shown to potentiate the algogenic effects of 5-HT but little is known about the mechanism behind this potentiation (Hong and Abbott, 1994). The data presented here demonstrates that PGE₂ is capable of sensitising a sub-population of cultured DRG neurones to low concentrations of 5-HT. PGE₂ pre-treatment caused a left shift of the 5-HT dose response

curve. The number of cells responding to higher concentrations of 5-HT did not significantly increase by pre-treatment with PGE₂.

The 5-HT concentration response curve was fitted to the Hill equation with a slope of n_H 0.36. This suggests that more than one receptor type mediates the response to 5-HT. However, pre-treatment with PGE₂ increased the sensitivity of DRG neurones to 5-HT resulting in a dose response curve with a Hill coefficient of 1. Note that the percentage of cells responding to 5-HT prior to and after pre-treatment plateau at a similar value. Thus, a possible explanation to fit the evidence above is that the calcium response to 5-HT is mediated by one receptor type present in two forms, unphosphorylated and phosphorylated.

PGE₂ pre-treatment did not have any effect on the characteristics of the $[Ca^{2+}]_i$ response elicited by 5-HT. Pre-treatment of the cells with forskolin produced potentiation that was similar to that produced by PGE₂. In these experiments intracellular cAMP was elevated by two independent methods yet both produced a significant increase in the number of cells responding to 5-HT. This confirms a significant role for cAMP in PGE₂ sensitisation of sensory neurones to 5-HT.

Pre-treatment of cells with H89 inhibited the effect of PGE₂ on the percentage of cells responding to 5-HT. This finding confirms the major role of PKA in the PGE₂ induced sensitisation of sensory neurones to 5-HT.

PKA involvement in the sensitisation mechanism supports the notion that the shift in the 5-HT dose response curve is due to the presence of one receptor type present in two forms. The dose response curve for 5-HT alone reflects the presence of both forms of the receptor. Pre-treatment of cells with PGE₂ is known to increase cAMP which in turn activates PKA. Therefore, PKA could then act on the unphosphorylated receptors increasing the proportion of receptors in the phosphorylated state.

These findings show that PGE₂ induced sensitisation of the response to 5-HT and histamine are mediated by PKA. A similar study showed that PGE₂ induced sensitisation of bradykinin response was also mediated by PKA (Smith *et al.*, 2000). This is supported by the findings from a study using mice with a targeted mutation of a PKA to investigate the role of PKA in modulating the sensitivity of sensory neurones to noxious thermal stimuli (Malmberg *et al.*, 1997). The heightened thermal sensitivity after injection of PGE₂ into the hindpaw of the targeted mice was reduced in comparison to the wild-type mice (Malmberg *et al.*, 1997).

Thus, a common mechanism appears to be responsible for the PGE₂ sensitisation of the responses to inflammatory mediators.

How does PKA induce sensitisation?

As shown above, the proinflammatory mediator PGE₂ can potentiate the responses of a number of inflammatory mediators, including histamine and

5-HT. In neurones sensitised with PGE₂ the characteristics of the time course of the [Ca²⁺]_i responses obtained to either histamine or 5-HT were unchanged. Thus, sensitisation is occurring upstream of calcium release. The leftward shift in the dose response curves to histamine and 5-HT shows a reduction in the threshold of activation. This is unlikely to be due to recruitment of previously unresponsive cells as the total number of cells responding to 5-HT or histamine before and after exposure to PGE₂ is similar. The fact that the sensitisation can be blocked by H89 is evidence for the involvement of PKA. This could occur through a number of mechanisms (see Figure 4.3):

1. Increased receptor affinity e.g. by phosphorylation of the receptor
2. Increase in receptor number as a result of insertion of preformed receptors into the plasma membrane.
3. Alteration of the G protein binding site, enhancing the sensitivity of the G protein to the receptor.

Consistent with my finding of a role for PKA, a recent study using Chinese hamster ovary cells expressing the human H₁ receptor showed that PKA activation induced significant phosphorylation of the H₁ receptor (Miyoshi *et al.*, 2004). In this case the authors could find no evidence for altered function but in this case they were looking for desensitisation rather than sensitisation.

Evidence in favour of the second mechanism is provided by a study on μ opioid receptors. In this study, stimulation of the cAMP/PKA pathway regulates the insertion of presynaptic receptors into the membrane of GABAergic terminals within the rat dorsal vagal complex (Browning *et al.*, 2004). Modulation of G protein binding is associated with the desensitisation of receptors (for review see Ferguson 2001). On the basis of current evidence, the first two mechanisms discussed above offer the most plausible explanation of the sensitisation. Nevertheless, the precise mechanism involved remains unresolved.

How could PKA induced sensitisation enhance cell excitability?

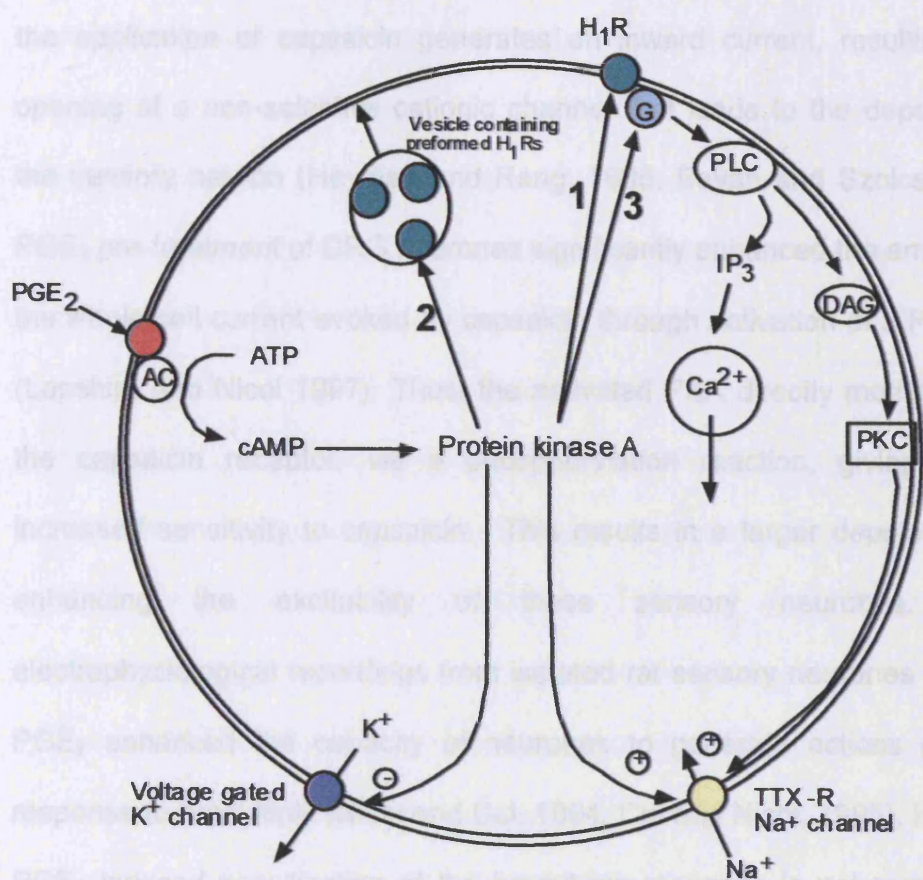
Sensitisation by inflammatory mediators increases the excitability of nerve endings resulting in increased firing of action potentials that are transmitted to the CNS. A number of studies have shown that application of PGE₂ to sensory neurones does increase the number of action potentials evoked by noxious thermal, chemical (e.g. elevated levels of potassium, bradykinin) and mechanical stimuli (Baccaglini and Hogan 1983, Martin *et al.*, 1987, Nicol and Cui 1994, Nicol *et al.*, 1997). This study has shown that the PGE₂ induced sensitisation of both the responses to histamine and to 5-HT are mediated by a cAMP-PKA mechanism. Although not covered by this study the following section discusses the possible mechanisms by which the PKA induced sensitisation of the responses to both agonists could lead to an enhanced excitability of cells, ultimately leading to the firing or increased firing of action potentials.

Figure 4.3 – The possible mechanisms employed by PKA to induce sensitisation and how PKA could enhance cell excitability.

The increased sensitivity of sensory neurones to 5-HT and histamine results from the activation of PKA. This could occur through one of three mechanisms (labelled on figure):

1. An increased potency and/or efficacy of agonist at the receptor e.g. as a result of receptor phosphorylation
2. Insertion of preformed receptors into the plasma membrane thus increasing the total number of available receptors.
3. An alteration of the G protein binding site, enhancing the sensitivity of the G protein to the receptor.

In addition it is known that PKA can regulate cell excitability through phosphorylation of sodium and potassium channels. The effects PKA has on these channels to enhance the cells excitability are shown in the lower part of the figure.



Electrophysiological recordings have shown that in responsive DRG neurones the application of capsaicin generates an inward current, resulting from the opening of a non-selective cationic channel that leads to the depolarisation of the sensory neuron (Heyman and Rang, 1985, Bevan and Szolcsanyi, 1990). PGE₂ pre-treatment of DRG neurones significantly enhanced the amplitude of the whole cell current evoked by capsaicin through activation of a PKA pathway (Lopshire and Nicol 1997). Thus, the activated PKA directly modifies a site on the capsaicin receptor, via a phosphorylation reaction, giving rise to an increased sensitivity to capsaicin. This results in a larger depolarisation thus enhancing the excitability of these sensory neurones. Moreover, electrophysiological recordings from isolated rat sensory neurones showed that PGE₂ enhanced the capacity of neurones to generate action potentials in response to bradykinin (Nicol and Cui, 1994, Cui and Nicol, 1995). However, the PGE₂ induced sensitisation of the bradykinin response is not mediated at the bradykinin receptor. Instead, the enhanced excitability is due to PKA induced modulation of other channels that contribute to the resting membrane potential (e.g. sodium and potassium channels) and thus regulate the capacity of neurones to initiate action potentials (Lopshire and Nicol 1997).

Nociceptive sensory neurones are known to express a wide variety of channels including at least two types of tetrodotoxin (TTX) resistant sodium channels, TTX-sensitive sodium channels as well as multiple types of voltage-gated calcium channels and potassium channels. Voltage gated sodium channels play an important role in the initiation and generation of action potentials in neurones.

Modulation of these channels can alter the properties of excitable cells and their responses to external and internal stimuli.

Activation of PKA by PGE₂ results in a dose-dependent increase in the amplitude of peak tetrodotoxin –resistant currents in adult and neonatal rat DRG sensory neurones (England *et al.*, 1996). The evidence that PKA inhibitors attenuated PGE₂ induced changes in TTX-R I_{Na} activation, inactivation and deactivation rates verified the involvement of PKA. The activation of PKA was believed to be the underlying mechanism of inflammatory mediator induced hyperalgesia and nociceptor sensitisation (Taiwo *et al.*, 1989, Cui and Nicol 1995). Moreover, it has now come to light that PKA's modulation of TTX-R I_{Na} is the underlying mechanism. Thus, the enhanced firing capacity seen with the application of bradykinin in the presence of PGE₂ is likely to result from the increase in the TTX resistant sodium current.

Studies using *in vivo* models showed increased kinase activity (PKA and/or PKC) in nociceptive C fibres during hyperalgesia (Dina *et al.*, 2001, Igwe and Chronwall 2001). Evidence from a number of studies have implicated a role for PKC in nociceptor sensitization (Cesare *et al* 1999, Tominaga *et al.* 2001).

Inhibitors of PKC significantly attenuated the PGE₂ induced modulation of TTX-resistant I_{Na} (Gold *et al.*, 1998). Thus, PKC activity is necessary for the expression of PGE₂ induced PKA modulation of TTX-R I_{Na}. The activation of particular E-type prostaglandin receptor subtypes may lead to activation of PKC.

In the case of the PGE₂ induced sensitisation of histamine and 5-HT responses, both agonists stimulate the IP₃/PLC cascade. This ultimately leads to the activation of PKC which could subsequently act on the TTX-resistant sodium channel. However, only one subtype of PGE₂ receptor activates PLC and my findings show no evidence that the application of PGE₂ increased intracellular calcium.

Additionally it has been shown that increasing levels of cAMP led to suppression of voltage dependent potassium currents in DRG neurones (Nicol *et al.*, 1997, Evans *et al.*, 1999). These voltage gated currents have the characteristics of a delayed rectifier-like current. The suppression of this current is dependent on the activation of the cAMP-PKA transduction cascade and would increase neuronal excitability. The possible mechanisms leading to increased neuronal excitability are summarised in Figure 4.3.

4.5 Conclusions

In this study DRG neurones have been used as a model for investigating the actions of pruritic and algogenic agents in primary sensory nerve endings. The cultured sensory neurons were derived from all levels of the spinal cord and are therefore of mixed origin. Furthermore, the transduction processes described here may differ from those of nerve endings in the skin. As the mechanisms described involve the release of calcium from internal stores, a key issue is whether the endoplasmic reticulum is present in fine sensory axons and whether the IP₃ receptor is also present. Several studies have provided evidence for the

existence of endoplasmic reticulum in axons (Ellisman and Lindsey, 1983; Terasaki et al., 1994; Aihara et al., 2001). Moreover, Aihara et al specifically localised GFP-tagged IP₃ receptors in the fine axons of cultured chick DRG neurons. It therefore is plausible that the findings discussed here will have some bearing on the peripheral mechanisms of the sensations of itch and pain.

The data presented here suggest that ATP, histamine and 5-HT all activate G protein linked receptors and that the subsequent generation of second messengers will influence the excitability of the nerve endings via effects on TTX resistant Na⁺ channels and potassium channels as discussed on pages 173-178. The excitation of the primary afferents is the first step of a complex process that lead to the conscious sensations of itch and pain. It is now generally accepted that there are specific pathways concerned with the transmission of pain. For itch the situation is less clear, and four distinct theories have been proposed (see McMahon & Koltzenburg, (1992). As mentioned in the Introduction, they are: i) the specificity theory which proposes that a specific set of afferents respond to pruritic stimuli; ii) the intensity theory which proposes that low levels of activity in nociceptive afferents signal itch while higher levels of activity signal pain; iii) the selectivity theory which proposes that there are no specific populations of afferents that signal itch, rather the sensation is mediated via a subset of nociceptive afferents that activate separate central connections responsible for the sensation of itch; iv) the pattern theory which proposes that itch is encoded by temporal or spatial discharge patterns in cutaneous afferents that also signal other modalities. The evidence reviewed in the Introduction

(p17) suggests that the intensity and pattern theories are unlikely explanations for the sensation of itch. This leaves the specificity theory and the selectivity theory to be evaluated.

As application of histamine to the superficial regions of the skin is known to elicit a pure sensation of itch (Magerl *et al.*, 1990; Ward *et al.*, 1996) itch can be considered to be a specific sensation. Moreover, the finding that some histamine sensitive DRG neurons are insensitive to algogenic agents such as capsaicin, ATP and 5-HT implies a degree of specificity in the response to histamine. However, it is well known that itch sensations can be masked by painful stimuli. It therefore seems that the specificity theory currently offers the most plausible explanation for the sensation of itch with the important proviso that a strong barrage of afferent information from pain afferent may block the transmission of pruritic information within the CNS.

4.6 Future research

This study has clarified the principal classes of receptor that mediate the responses to 5-HT and ATP. As shown in the discussion, each receptor class contains multiple types of subtypes and the Hill coefficient obtained from the dose-response curves suggested that both the responses to 5-HT and ATP probably involved multiple receptor subtypes. Receptor subtype selective agonists and antagonists (where available) could be used to further investigate the receptors subtypes involved. Characterisation of the 5-HT₂ receptor subtypes could be further investigated with the 5-HT_{2A} antagonist, ketanserin.

However, as the selectivity of the antagonists is not always reliable, *in situ* hybridisation and binding studies could be also used to help clarify exactly which receptors are present and whether they are functional. Immunohistochemistry could also be used to determine the location of different receptor subtypes on the DRG neurones and on the primary afferents. This technique could also be used to examine whether the receptors for the various inflammatory mediators were co-localised.

This study additionally investigated the PGE₂ induced sensitisation of the [Ca²⁺]_i responses to histamine and 5-HT. PKA was found to mediate this sensitisation however it is not known how this occurs. The neuronal protein, which is phosphorylated by PKA, whether this is an ion channel, part of the receptor or a G protein remains to be resolved. Currently, it is not known if the responses to ATP can also be sensitised and if this is the case, whether PKA also mediates this sensitisation.

Once the receptor subtypes and transduction processes have been clarified, the next logical step would be to investigate how selective activation of the different receptor subtypes influences the excitability of the sensory neurons.

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6.0 APPENDIX

6.1 DRUGS AND SOLUTIONS

Unless otherwise stated all laboratory drugs and chemicals were obtained from Sigma-Aldrich Company Ltd. (The Old Brickyard, New Road, Gillingham, Dorset SP8 4XT).

Alpha Diagnostic International (5415 Lost Lane, San Antonio, TX 78238, USA)

Rat Histamine 1 Receptor (H₁R) Antibody, Rat H₁R control peptide.

Alomone Laboratories (Shatner Centre 3, PO Box 4287, Jerusalem 91042, Israel).

Mouse Nerve Growth Factor (mNGF 2.5s) Grade 1.

Bio-Rad Laboratories Ltd (Marylands Avenue, Hemel Hempstead, Hertfordshire) 4-15% gradient gels

Calbiochem Novabiochem (Merck Biosciences Ltd. Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR)

Forskolin (7b-ace, 1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-amino)hexyl]-1H-pyrrole-2,5-dione (U73122)

Cayman Chemicals (1180 East Ellsworth Road, Ann Arbor, MI 48108, USA)

Prostaglandin E₂

Invitrogen (3 Fountain Drive, Inchinnan Business Park, Paisley, UK) L-Glutamine, Penicillin, Streptomycin, Laminin, Benchmark pre-stained protein ladder

Jackson Immuno Research Labs (P.O. Box 9, 872 West Baltimore Pike, West Grove, PA, USA) Donkey anti rabbit fluorescein isothiocyanate (FITC), Donkey anti rabbit/goat tetramethyl rhodamine isothiocyanate (TRITC)

JRH Biosciences (13804, West 107th Street, Lenexa, KS, USA) Ham's nutrient mixture F14 modified with L-Glutamine and 1.176g/L sodium bicarbonate

Molecular Probes (Cambridge bioscience, 24/25 Signet Court, Newmarket Road, Cambridge, CB5 8LA)
Fura-2 acetoxymethyl ester, Pluronic acid

Tocris Cookson (Northpoint, Fourth Way, Avonmouth, Bristol, BS11 8TA)
 α -Methyl-5-hydroxytryptamine maleate, m-Chlorophenylbiguanide hydrochloride, 2-Methylthioadenosine triphosphate tetrasodium,

Solutions

Culture Media

To each 500 ml bottle of Ham's F14 was added 1 mM L-Glutamine, 5 mls penicillin-streptomycin (5000 IU/ml penicillin and 5000 µg/ml streptomycin) and 20 mls USG (to give 4%)

Buffer Solution

HEPES buffered Na Locke

NaCl 140 mM, KCl 5mM, Mg Cl₂ 1 mM, Ca Cl₂ 2 mM, HEPES 15 mM
Glucose 5.5 mM.

HEPES buffered High K⁺ Locke

NaCl 50 mM, KCl 95 mM, Mg Cl₂ 1 mM, Ca Cl₂ 2 mM, HEPES 15 mM
Glucose 5.5 mM.

HEPES buffered 0 mM calcium Na Locke

NaCl 140 mM, KCl 5 mM, Mg Cl₂ 1 mM, HEPES 15 mM Glucose 5.5 mM.

Western Blot Solutions

5X Running Buffer

15.1 g Tris Base, 94 g Glycine, 50 mls 10% SDS- final volume 1 litre

10X Transfer Buffer

72 g Glycine, 15 g Tris Base – final volume 1 litre

Before use 100 mls 10X transfer buffer, 200 mls menthanol, 5 mls 10% SDS

– final volume 1 litre

Loading Buffer

187.5 mM tris HCl pH 6.8, 30% glycerol, 6% SDS, 0.1% Bromophenol blue, 150 mM DTT, 1:10 mercaptoethanol.

TBST (Tris Buffered Saline Tween) Buffer

150 mM NaCl, 25 mM Tris HCl pH 7.4, 0.5 mls Tween 20 – final volume 1 litre.

Lysis Buffer

50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1% SDS, Aprotinin (1:100 stock from sigma diluted), 1 mM PMSF, 1 µg/ml pepstatin.

Immunohistochemistry**Antibody Buffer Solution**

Phosphate buffered saline, triton x100, 0.02% sodium azide.

Mounting Medium

Phosphate buffered saline / Glycerol (1:3) containing 25 mg/ml diazobicyclo-2-2-octane.

Blocking solution

10% Marvel in phosphate buffered saline.

6.2 – Western Blot Protocol

Sample Preparation

- Suspend the dorsal root ganglion neurones in lysis buffer (see appendix 6.1) and homogenised using a clean micropestle.
- Spin the sample then remove the supernatant to a fresh eppendorf and add loading buffer (one third of the total volume).

Preparation of Gradient SDS Gels

- Assemble gels in the gel apparatus with small plates facing inwards and clamp onto mount to form tight seal.
- Pour running buffer into the chamber between gels covering the wells then pour the remaining buffer into the external tank.
- Using fine gel loading pipette tips load equal volumes of sample (30 μ l –50 μ l) to each well.
- Load the pre-stained molecular weight markers into the reference lanes.
- Run gel at a constant 100V for 1.5-2 hours.

Transfer of Gel

- Dismantle the gel tank. Check the markers have transferred
- Soak sponges, filter paper, nitrocellulose membrane in transfer buffer. Assemble on transfer cassette in following order – sponge, filter paper, gel, nitrocellulose membrane, filter paper and finally sponge.
- Try to remove any air bubbles before closing the cassette and placing in the tank.

- Place ice block next to transfer apparatus, fill tank with transfer buffer and run transfer at constant 0.4A for 1.5-2 hours.

Immunoblotting

- Dismantle the transfer apparatus and remove the membrane, cut the top right hand corner to aid orientation later.
- Place membrane in small tray containing 10% milk solution, cover with cling film and leave on a rocker for one hour at room temperature.
- .After an hour replace the 10% milk solution with a 5% milk solution containing the required primary antibody at an appropriate dilution.
- Cover the tray with cling film and leave on a rocker is covered and leave on a rocker overnight at 4°C.
- Wash membrane three time for fifteen minutes each time in a large volume of TBST (see appendix 6.1)
- Place membrane in tray containing the secondary antibody diluted to the required concentration in 5% milk solution. Covered with cling film and place on a rocker at room temperature for 1-2 hours.
- Wash membrane three time for fifteen minutes each time in a large volume of TBST (see appendix 6.1)

Chemiluminescent Detection

- Mix equal volumes of the detection reagent 1+2 in a tube to give an adequate working volume.

- Remove blots from the final TBST wash and place membrane protein side up on a large piece of cling film.
- Pipette the ECL working reagent directly onto the membrane and leave at room temperature for 2-3 minutes.
- Blot off the excess solution, cover the membrane with cling film, making sure there are no creases, and place in xray cassette.
- In dark room expose the blot to the Xray film for a short period of time (1-2 minutes).
- To develop the exposed film immerse quickly into developing solution, wash in water, transfer to fixer and finally wash. Hang up to dry.
- When dried compare the bands on the X film with the molecular weight markers on the blot to give an estimation of the size of the proteins obtained.